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(54) **Title:** RNA INTERFERENCE THAT BLOCKS EXPRESSION OF PRO-APOPTOTIC PROTEINS POTENTIATES IMMUNITY INDUCED BY DNA AND TRANSFECTED DENDRITIC CELL VACCINES

(57) **Abstract:** An immunotherapeutic strategy is disclosed that combines antigen-encoding DNA vaccine compositions combined with siRNA directed to pro-apoptotic genes, primarily Bak and Bax, the products of which are known to lead to apoptotic death. Gene gun delivery (particle bombardment) of siRNA specific for Bak and/or Bax to antigen-expressing DCs prolongs the lives of such DCs and lead to enhanced generation of antigen-specific CD8+T cell-mediated immune responses *in vivo*. Similarly, antigen-loaded DCs transfected with siRNA targeting Bak and/or Bax serve as improved immunogens and tumor immunotherapeutic agents.

RNA Interference that Blocks Expression of Pro-Apoptotic Proteins Potentiates Immunity Induced by DNA and Transfected Dendritic Cell Vaccines

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention in the fields of molecular biology, immunology and medicine relates to combinations or mixtures of nucleic acid molecules and chimeric nucleic acid molecules that encode an antigen and a small interfering RNA (siRNA). The expression of the siRNA blocks expression of one or more an anti-apoptotic protein *in vivo*. This results in prolonging the life of important antigen presenting cells, dendritic cells (DCs), and as a consequence, the more potent induction and enhancement immune responses, primarily cytotoxic T lymphocyte (CTL) responses to specific antigens such as tumor or viral antigens.

Description of the Background Art

Cytotoxic T lymphocytes (CTL) are critical effectors of anti-viral and antitumor responses (reviewed in Chen, CH *et al.*, J Biomed Sci. *5:* 231-252, 1998; Pardoll, DM. *Nat Med. 4:* 525-531, 1998; Wang, RF *et al., Immunol Rev. 170:* 85-100, 1999). Activated CTL are effector cells that mediate antitumor immunity by direct lysis of their target tumor cells or virus-infected cells and by releasing of cytokines that orchestrate immune and inflammatory responses that interfere with tumor growth or metastasis, or viral spread. Depletion of $CD8^+$ CTL leads to the loss of antitumor effects of several cancer vaccines (Lin, K-Y *et aL, Cane Res 56:* 21-26, 1996; Chen, C-H *et aL, Cane Res. 60:* 1035-42, 2000). Therefore, the enhancement of antigen presentation through the MHC class I pathway to $CD8^+$ T cells has been a primary focus of cancer immunotherapy.

Naked DNA vaccines have emerged recently as attractive approaches for vaccine development (reviewed in Hoffman, SL *et aL, Ann N YAcad Sci 772:* 88-94, 1995; Robinson, HL. *Vaccine 15:* 785-787, 1997; Donnelly, JJ *et aL, Annu Rev Immunol 15:* 617-648, 1997; Klinman, DM *et aL, Immunity 11:* 123-129, 1999; Restifo, NP *et aL, Gene Ther 7:* 89-92, 2000; Gurunathan, S *et al.,Annu Rev Immunol 18: 921-91A,* 2000). DNA vaccines generated long-term cell-mediated immunity (reviewed in Gurunathan, S *et aL, Curr Opin Immunol 12:* 442-447, 2000) and can generate $CD8^+$ T cell responses in vaccinated humans (Wang, R *et al. Science 282:* 476-480, 1998).

However, one limitation of these vaccines is their lack of potency, since the DNA vaccine vectors generally do not have the intrinsic ability to be amplified and to spread *in vivo* as do some replicating viral vaccine vectors. Furthermore, some tumor antigens such as the E7 and E6 proteins of human papillomavirus- 16 ("HPV- 16") are weak immunogens (Chen et al., 2000, *supra).* Therefore,

there is a need in the art for strategies to enhance DNA vaccine potency, particularly for more effective cancer and viral immunotherapy.

The present inventors and their colleagues demonstrated that linkage of HPV-16 E7 antigen to a number of immunogenicity-potentiating polypeptides (Kim JW *et al, Gene Ther. 11:* 101 1-18, 2004,), such as *Mycobacterium tuberculosis (Mtb)* heat shock protein 70 (Hsp70) (Chen *et al, supra;* Wu *et al,* WO 01/29233) and CRT (Cheng WF *et al, J Clin Invest,* 2001, 705:669-78; WO/0212281) result in the enhancement of DNA vaccine potency. See, also Cheng WF *et al, Vaccine* 23:3864-74, 2005; Peng S *et al, JBiomed ScL* 72:689-700, 2005; Peng S *et al, J Virol.* 2004, 75:8468-76; Peng S *et al, Gene Ther.* 2005 (Sep 22; Epublished ahead of print)

Others have shown, using protein vaccines, as distinct from DNA immunogens, that immunization with HSP complexes isolated from tumor or virus-infected cells potentiated anti-tumor immunity (Janetzki, S *et al, J Immunother 21:269-7,* 1998) or antiviral immunity (Heikema, A *et al, Immunol Lett 57:69-14,* 1997). Immunogenic HSP-peptide complexes could be reconstituted *in vitro* by mixing the peptides with HSPs (Ciupitu, AM *et al,* 1998. *J Exp Med 187:685-9,* 1998). HSP-based protein vaccines have been created by fusing antigens to HSPs (Suzue, K *et al, J Immunol* 75(5:873-79, 1996). However, prior to the discoveries of the present inventors and their colleagues since about 1999 with DNA immunogens, HSP vaccines (and those employing other intracellular transport proteins or intercellular spreading proteins) were limited to peptide/protein molecules that were typically produced bacteria using bacterial expression vectors and purified therefrom. The present inventors and their colleagues were the first to provide naked DNA and self-replicating RNA vaccines that incorporated HSP70 and other immunogenicity-potentiating polypeptides. The present inventors and their colleagues were also the first to demonstrate that linking antigen to intracellular targeting moieties calreticulin (CRT), domain π of *Pseudomonas aeruginosa* exotoxin A (ETA(dII)), or the sorting signal of the lysosome-associated membrane protein type 1 (Sig/LAMP-1) enhanced DNA vaccine potency compared to compositions comprising only DNA encoding the antigen of interest. To enhance MHC class II antigen processing, one of the present inventors and colleagues (Lin, KY *et al,* 1996, *Cane Res 56: 21-*26) linked the sorting signals of the lysosome-associated membrane protein (LAMP-I) to the cytoplasmic/nuclear human papilloma virus (HPV-16) E7 antigen, creating a chimera (Sig/E7/LAMP-1). Expression of this chimera *in vitro* and *in vivo* with a recombinant vaccinia vector had targeted E7 to endosomal and lysosomal compartments and enhanced MHC class II presentation to CD4+ T cells. This vector was found to induce *in vivo* protection against an E7+ tumor, TC-I so that 80% of mice vaccinated with the chimeric Sig/E7/LAMP1 vaccinia remained tumor free 3 months after tumor injection. Treatment with the Sig/E7/LAMP-1 vaccinia vaccine cured mice with small established TC-I tumors, whereas the wild-type E7-vaccinia showed no effect on this established tumor burden. These

findings point to the importance of adding an immunopotentiating "element" (in the form of DNA encoding that "element") to DNA encoding an antigen to enhance *in vivo* potency of a recombinant DNA vaccine for antigens that are presented as either MHC class I- or MHC class II- antigen complexes, such as by rerouting a cytosolic tumor antigen to the endosomal/lysosomal compartment.

Intradermal administration of DNA vaccines via gene gun can efficiently deliver genes of interest into professional antigen presenting cells (APCs) *in vivo* (Condon C *et al, Nat Med, 2:* 1122-28, 1996). The skin contains numerous bone marrow-derived APCs (called Langerhans cells) that are able to move through the lymphatic system from the site of injection to draining lymph nodes (LNs), where they can prime antigen-specific T cells (Porgador A *et al., JExp Med 188:* 1075-1082, 1998). Powerful APCs in other sites, particularly in lymphatic tissue are dendritic cells (DC). Gene gun immunization therefore provides the opportunity to test vaccine strategies that require direct delivery of DNA or RNA to APCs.

Antigen presentation by DCs is a critical element for the induction of the cellular immune responses that mediate various types of immunotherapy, particularly tumor immunotherapy. Several studies demonstrated that immunization with tumor antigen-pulsed DCs could break the tolerance of the immune system against antigens expressed by tumor cells and in some cases generate appreciable clinical responses. Thus, DC-based vaccines represent a promising method for the treatment of malignancies. See, for example, Gunzer, M *et al, Crit Rev Immunol 21:* 133-45, 2001; Engleman, EG Dendritic cell-based cancer immunotherapy. *Semin Oncol 30:23-29,* 2003; Schuler, G *et al, Curr Opin Immunol* 75:138-147, 2003; Cerundolo, V *et al.,* Dendritic cells: a journey from laboratory to clinic. *Nat Immunol* 5:7-10, 2004; Figdor, CG *et al., Nat Med* 70:475-480, 2004; Markiewicz, MA *et al., Cancer Invest* 22:417-434, 2004; Turtle, CJ *et al, Curr Drug Targets* 5:17-39, 2004).

Dendritic cell-based vaccines have become an important approach for the treatment of malignancies. Numerous techniques have recently been designed to optimize dendritic cell activation, tumor antigen delivery to dendritic cells, and induction of tumor-specific immune responses *in vivo.* Dendritic cells, however, have a limited life span because they are subject to apoptotic cell death mediated by T cells, hindering their long-term ability to prime antigen-specific T cells.

DCs, however, have a limited life span that hinders their long-term ability to prime antigen-specific T cells (see Ronchese, F *et al. J Exp Med 194:F23-26,* 2001). A principal contributor to the shortened lifespan of DCs is CTL-induced apoptosis. After activation by DCs, CTLs that recognize epitopes can kill target cells expressing these epitopes, typically presented by MHC Class I proteins. Because DCs express MHC-I:antigen peptide complexes, newly primed CTLs can kill the very DCs that activated them (Medema, JP *et al, J Exp Med 194:657-667,* 2001). Thus, DC-based vaccination should be enhanced by inhibiting apoptosis and prolong survival of antigen-expressing DCs *in vivo* (Kim, TW *et*

al, J Immunol *171:2970-2976,* 2003a; Kim, TW *et al, J Clin Invest 112:* 109-17, 2003(b); and a patent application by the present inventors and colleagues WO05/047501 (26-MAY-05) incorporated herein by reference in its entirety.

The present inventors and their colleagues have used gene gun immunization of DNA compositions to test vaccine strategies that involve intracellular targeting strategies that direct delivery of DNA or RNA to APCs. The targeting molecules (using coding DNA linked to DNA encoding an antigen) that have shown potent effects include *Mycobacterium tuberculosis* heat shock protein 70 (HSP70) (Chen CH *et al., 2000, Cancer Res* 60:1035-42, 2000), calreticulin (CRT; Cheng WF, 2001, *supra),* and the sorting signal of the lysosome-associated membrane protein 1 (LAMP-I; Ji H *et al, Hum Gene Tlierapy, 10:2727-40,* 1999).

Vaccination with DNA vectors that encode such fusion proteins are able to route an antigen (generally exemplified with HPV-16 E6 and E7) to desired subcellular compartments, and enhance antigen processing and presentation to T cells. Therefore, direct delivery of DNA vaccines into DCs via gene gun provides an opportunity to modify the quality and quantity of DNA-transfected DCs and influence vaccine potency.

T cell-mediated apoptotic cell death can occur through two major pathways, the intrinsic and the extrinsic pathways. See, for example, Russell, JH *et al.,Annu Rev Immunol* 20:323-370, 2002). In general, death domain-containing receptors such as CD95 (APO-I/Fas) can sense the external signal (such as Fas ligand) and activate the extrinsic apoptotic pathway through the Fas-associated death domain (Fadd). This pathway is mediated by recruitment and activation of caspase-8, an initiator caspase, in the death-inducing signaling complex (DISC) followed by direct cleavage of downstream effector caspases.

The intrinsic pathway (granzyme B/perforin-mediated apoptosis), important for T cell-mediated induction of apoptotic DC death, initiates from within the cell. The pore-forming protein perform and the serine protease granzyme B secreted into cells by antigen-specific $CD8^+$ T cells induce intracellular changes, such as DNA damage, resulting in the release of a number of pro-apoptotic factors from mitochondria, such as cytochrome c, leading to the activation of another initiator caspase, caspase-9 (Jacotot, E *et al, Ann N YAcad Sci 887:* 18-30, 1999; Korsmeyer, SJ *et al, Cell Death Differ 7:* 1166-73, 2000; Degli Esposti, M *et al,* Dive, C. *Biochem Biophys Res Commun 30*ΦA*55-61,* 2003; Opferman JT *et al, Nat Immunol 4:* 410-15, 2003). 5-61, 2003; Opferman JT *et al, Nat Immunol 4:* 410-15, 2003). Activated caspase-9 leads to the activation of effector caspases (caspase-3, -6, and -7) in a protein complex called the apoptosome (for review, see Johnson, CR *et al, Apoptosis* 9:423-27, 2004) leading to proteolysis of a cascade of substrates and apoptotic death.

Thus Bak, Bax, and caspase 9 are clearly important pro-apoptotic proteins for the intrinsic apoptotic pathway and caspases-8 and -3 are is an important pro-apoptotic proteins in the extrinsic apoptotic pathway. Because of the role of Bak and Bax as gatekeepers in the intrinsic apoptotic pathway, the present inventors have conceived of targeting these genes for inhibition by RNA interference (RNAi) to diminish DC apoptosis. This is disclosed in detail and exemplified below. However, the present inventors conception includes a similar targeting of caspase-9, caspase-3 and caspase-8.

RNA interference (RNAi) is a recently reported phenomenon that has developed into a new approach for elucidating and regulating gene function. RNAi is a sequence-specific, post-transcriptional, gene-silencing mechanism that is effected through double-stranded RNA (dsRNA) molecules homologous to a sequence of the target gene (Elbashir, SM *et al, Nature 411:494-498,* 2001; Fire, *h et al, Nature* 397:806-811, 1998; Tuschl, T *etal, Genes Dev* 73:3191-3197, 1999). Fragments of the dsRNA called "small interfering" RNAs (siRNAs) can rapidly induce loss of function, and only a few molecules are required in a cell to produce the effect (Fire *et al, supra)* through hybrid formation between a homologous siRNA and mRNA (Lin, SL *et al, Curr Cancer Drug Targets* 7:241-247, 2001). A member of the RNase HI family of nucleases named *dicer* has been identified as being involved in processing (Bernstein, E *et al, Nature 409:363-366,* 2001). DNA vector-mediated RNAi technology has made it possible to develop therapeutic applications for use in mammalian cells (Sui, G *et al, Proc Natl Acad Sci USA* 99:5515-5520, 2002; McCaffrey, AP *et al, Nature 418:38-39,* 2002; Lee, NS *et al, Nat Biotechnol* 20:500-505, 2002). There have been several reports of delivery of siRNA by retroviral vectors for stable expression (Barton, G.M *et al, Proc Natl Acad Sci USA 99:* 14943-14945, 2002; Paddison, PJ *et al, Cancer Cell* 2:17-23, 2002; Rubinson, DA *et al, Nat Genet* 33:401-406, 2003; Tiscornia, G *etal, Proc Natl Acad Sci USA* 700:1844-1848, 2003) or by adenoviral vectors for transient expression (Xia, H *et al, Nat Biotechnol 20:* 1006-1010, 2002). RNAi may be effected by small interfering RNA molecules (siRNA) that induce sequence-specific degradation of mRNA or by inhibiting translation of its complementary mRNA (see, for example, Mittal V. *Nat Rev Genetics* 5:355-65, 2004). Use of this approach to prolong the life of DCs by targeting pro-apoptotic proteins with the appropriate siRNAs is one of the objects of the present invention.

SUMMARY OF THE INVENTION

Partial List of Abbreviations used

APC, antigen presenting cell; BM, bone marrow; BM-DC, BM-derived dendritic cells; CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; CRT, calreticulin; DC, dendritic cell; E6, HPV oncoprotein E6; E7, HPV oncoprotein E7; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; HPV, human papillomavirus; HSP, heat shock protein; Hsp70, mycobacterial heat shock protein 70; IFNγ, interferon-γ, i.m., intramuscular(ly); i.v., intravenous; IPP, immunogenicity-potentiating (or -promoting) polypeptide; LN, lymph node; MHC, major histocompatibility complex; PBS,

phosphate-buffered saline; PCR, polymerase chain reaction; RNAi, RNA interference or interfering RNA; siRNA, small interfering RNA; siNA, small interfering nucleic acid; β-gal, β-galactosidase.

The present inventors have designed and disclose herein an immunotherapeutic strategy that combines antigen-encoding DNA vaccine compositions with siRNAs directed to pro-apoptotic genes, primarily Bak and Bax, the products of which are known to lead to apoptotic death of, *inter alia,* DCs. The present inventors conceived that gene gun delivery (particle bombardment) or delivery by other appropriate routes of siRNA specific for Bak and/or Bax to antigen-expressing (antigen-presenting) DCs would prolong the lives of such transfected DCs and lead to enhanced generation of antigen-specific T cell-mediated immune responses *in vivo.*

The present disclosure shows the impact of intradermal (gene gun) coadministration of DNA vaccines encoding HPV-16 E7 antigen with Bak and/or Bax siRNA. The present inventors chose HPV-16 E7 as a model antigen because HPVs, particularly HPV-16, are associated with a majority of cervical cancers, and E7 (and E6) is essential for oncogenic cell transformation. Use of constructs comprising DNA encoding HPV protein E6 would be expected to have the same activity (as supported by comparisons between the two using the present inventors' other immunopotentiating strategies). Minimally genetically modified E7 or E6 proteins ("detox") which have been rendered incapable of oncogenic activity by between 1 and 3 point mutations may be used in place of wild-type E7 and E6, and are safer for human subjects.

Effective vaccines against E7 (and/or E6) can be used to control HPV infections and HPV-associated lesions. As disclosed herein, evaluation of E7-specific immune responses, antitumor effects, and survival of DNA-transfected DCs, confirmed the present inventors' conception that co¬ administration of (i) a DNA vaccine or immunogen comprising sequences encoding an antigen with Bak- and/or Bax-specific siRNA (which term is used interchangeably with "Bak- or Bax siRNA") is a successful and innovative strategy for enhancing DNA vaccine potency.

As disclosed in herein (see Examples 8 *et seq.)* in DCs that are transfected with Bak/Bax siRNA Bak and Bax protein expression is abolished. According to the present invention, DCs transfected with Bak and Bax siRNA that are pulsed (loaded) with an antigenic peptide, so that they present that peptide, induce more potent antigen-specific $CD8^+$ T cell immune responses and antitumor effects in vaccinated subject mice, compared to peptide-pulsed DCs transfected with control siRNA. Bak/Bax siRNA-transfected DCs survive better *in vivo* than do antigenic peptide-loaded DCs transfected with a control siRNA in mice into which antigen-specific $CD8^+$T cells (able to kill the antigen-presenting DCs) have been adoptively transferred. Bone marrow-derived DCs (BM-DCs) and long-term DC cell lines as shown to be useful celllular immunogens.

The foregoing conceptions and discoveries provide a basis for clinical therapy of pathologies associated with any antigen, such as an antigen from a pathogenic microorganism (virus, bacterium, parasite), and pathogenic "endogenous" cells such as a tumor or cancer cells. Examples of viral antigens against which this strategy is exemplified herein are the two oncoprotein antigens from HPV-16, namely E6 and E7.

The invention exploits siRNA-based strategies to manipulate the functions, primarily to promote the survival, of DCs exposed to the siRNA *ex vivo* and/or *in vivo*. The siRNA-encoding constructs described can be used in combination with the strategy of enhancing the presentation of antigen through the MHC class I pathway to $CD8^+$ T cells by exploiting the features of certain polypeptides to target or translocate the antigenic polypeptide to which they are fused. Such polypeptide are referred to collectively herein as "/mmunogenicity-p_otentiating (or -promoting) polypeptide" or "IPP" to reflect this general property, even though these IPP's may act by any of a number of cellular and molecular mechanisms that may or may not share common steps. This designation is intended to be interchangeable with the term "targeting polypeptide." Inclusion of nucleic acid sequences that encode polypeptides that modify the way the antigen encoded by molecular vaccine is "received" or "handled" by the immune system serve as a basis for enhancing vaccine potency. All of these polypeptides in some way, contribute to the augmentation of the specific immune response to an antigen to which they are linked by one or another means that these molecules "employ" to effect the way in which the cells of the immune system handle the antigen or respond with cell proliferation and/or survival. IPP's may be produced as fusion or chimeric polypeptides with the antigen, or may be expressed from the same nucleic acid vector but produced as distinct expression products.

In addition to the strategy of including DNA encoding such IPPs in their vaccine constructs, the present invention harnesses the additional biological mechanism of inhibiting apoptosis by employing the RNAi approach significantly enhances T cell responses to DNA vaccine comprising antigen-coding sequences (with or without linked sequences encoding such IPPs).

Intradermal vaccination by gene gun efficiently delivers a DNA vaccine into DCs of the skin, resulting in the activation and priming of antigen-specific T cells *in vivo*. DCs, however, have a limited life span, hindering their long-term ability to prime antigen-specific T cells. According to the present invention, a strategy that prolongs the survival of DNA-transduced DCs enhances priming of antigen-specific T cells and thereby, increase DNA vaccine potency. As described herein co-delivery of siRNA that suppresses the expression of apoptotic pathways via Bak and Bax, prolongs the survival of transduced DCs. More importantly, vaccinated subjects exhibited significant enhancement in antigen-specific CD8+ T cell immune responses, resulting in a potent antitumor effect against antigen-expressing tumors. In another embodiment, instead of delivering the siRNA directly via gene gun, DNA encoding

the siRNA is delivered either as part of the same vector that encodes the antigen, or as a separate vector that is co-administered.

The combination of a strategy to prolong DC life (via siRNA) with intracellular targeting strategies afforded by certain EPPs produces a more effective DNA vaccine against E7, E6 or any antigen. Co-administration of siRNA (or DNA encoding siRNA) directed to Bak and/or Bax with DNA encoding antigen (exemplified as E7) linked to DNA encoding HSP70, CRT, or Sig/LAMP-1 results in further enhancement of the antigen (here E7)-specific CD8+ T cell response for all three types of constructs. This combination increases CD8+ T cell functional avidity, and increases the E7-specific CD4+ ThI cell response, enhances tumor therapeutic effect, and will yield more durable tumor protection when compared with mice vaccinated without the siRNA. Therefore, DNA vaccines that combine strategies to enhance intracellular antigen processing and prolong DC life have clinical utility for control of viral infection and neoplasia, among other forms of pathology where immunotherapy is useful as an ameliorative or curative therapy.

Thus, the present invention is directed to a nucleic acid composition useful as an immunogen, comprising a combination of:

(a) a first nucleic acid molecule comprising a first sequence encoding an epitope of an antigenic polypeptide or peptide; and optionally, linked to the first sequence, directly or via a linker, a second sequence that encodes an immunogenicity-potentiating polypeptide (D?P); and

(c) a second nucleic acid molecule the activity or expression of which stimulates development of an immune response to the epitope, which second nucleic molecule is (i) a siNA or (ii) DNA that encodes the siNA, wherein the siNA has a sequence that is sufficiently complementary to, and thus targets, the sequence of mRNA that encodes a pro-apoptotic protein expressed in a dendritic cell (DC), such that the activity or expression of the siNA in the cell results in inhibition of or loss of expression of the mRNA, resulting in inhibition of apoptosis and increased survival of DCs,

wherein the development of the immune response is stimulated.

The IPP above is preferably fused in frame to the first sequence such that the first and the second sequences encode a fusion protein comprising the antigenic epitope and the D?P. The IPP acts in potentiating an immune response preferably by promoting:

(a) processing of the linked antigenic polypeptide via the MHC class I pathway or targeting of a cellular compartment that increases the processing;

(b) development, accumulation or activity of antigen presenting cells or targeting of antigen to compartments of the antigen presenting cells leading to enhanced antigen presentation;

(c) intercellular transport and spreading of the antigen; or

(d) any combination of (a)-(c).

In the above composition, the IPP is: preferably

(a) the sorting signal of the lysosome-associated membrane protein type 1 (Sig/LAMP-1)

(b) a mycobacterial HSP70 polypeptide, the C-terminal domain thereof, or a functional homologue or derivative of the polypeptide or domain;

(c) a viral intercellular spreading protein selected from the group of herpes simplex virus-1 VP22 protein, Marek's disease virus UL49 protein or a functional homologue or derivative thereof;

(d) an endoplasmic reticulum chaperone polypeptide selected from the group of calreticulin or a domain thereof, ER60, GRP94, gp96, or a functional homologue or derivative thereof.

(e) domain II of *Pseudomonas* exotoxin ETA or a functional homologue or derivative thereof;

(f) a polypeptide that targets the centrosome compartment of a cell selected from γ-tubulin or a functional homologue or derivative thereof; or

(g) a polypeptide that stimulates DC precursors or activates DC activity selected from the group consisting of GM-CSF, Flt3-ligand extracellular domain, or a functional homologue or derivative thereof.

In the above composition the pro-apoptotic protein is preferably selected from the group consisting of one or more of (a) Bak, (b) Bax, (c) caspase-8, (d) caspase-9 and (e) caspase-3. Most preferably the siNA targets the encoding mRNA of anti-apoptotic protein Bak and/or Bax.

Preferably the siNA is an siRNA. The siRNA preferably targets SEQ ID NO:4 of Bak and/or SEQ ID NO:8 of Bax. The preferred siRNA is selected from the group consisting of(a) EQ ID NO:1/SEQ ID NO:2; and(b) SEQ ID NO:5/SEQ ID NO:6.

The antigenic polypeptide or peptide of the above composition preferably comprises an epitope that binds to and is presented on surfaces of antigen-presenting cells by MHC class I proteins. The epitope may be between about 8 and about 11 amino acid residues in length.

The antigenic polypeptide or peptide of the above composition preferably (i) is derived from a pathogen such as a mammalian cell *(e.g.,* specific or tumor-associated antigen), a microorganism or a virus; or (ii) cross-reacts with an antigen of the pathogen; or (iii) is expressed on the surface of a pathogenic cell. Preferred antigens are from a human papilloma virus, primarily the E7 and E6 polypeptide, including the "detox" forms of these polypeptides.

In the above composition, the first nucleic acid molecule is preferably an expression vector comprising a promoter operatively linked to the first and/or the second sequence; the promoter is preferably one that is expressed in an APC, most preferably in a DC.

Also provided herein are particles comprising a material suitable for introduction into a cell or an animal by particle bombardment to which particles is bound the above composition. Also intended is a combination of first and second particles each comprising a material is suitable for introduction into a cell or an animal by particle bombardment, and to which particles is bound the above composition. Wherein (a) the first nucleic acid molecules are bound to a first set of particles; and (b) the second nucleic acids (the siNA, preferably siRNA) are bound to a second set of particles. The preferred particles are gold particles.

This invention includes a pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising any of the above compositions or particles and a pharmaceutically acceptable carrier or excipient.

In another embodiment, the invention is directed to a method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the above composition or particles thereby inducing or enhancing the antigen specific immune response. The antigen specific immune response is preferably one mediated at least in part by $CD8^+$ cytotoxic T lymphocytes (CTL). The method is preferably carried out on a mammalian, most preferably a human subject. In the method, the composition or particles are preferably administered intradermally by particle bombardment (gene gun). The composition may also be administered intratumorally or peritumorally.

One embodiment is directed to a method of increasing the numbers of $CD8^+$ CTLs specific for a selected desired antigen in a subject comprising administering an effective amount of the above composition, particles or pharmaceutical compositions, wherein the antigenic peptide or polypeptide comprises an epitope that binds to and is presented on surfaces of APCs by MHC class I proteins.

Also provided is a method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of the above composition, particles or pharmaceutical compositions, wherein the antigenic epitopes are those expressed by the tumor or ones cross reactive with those expressed by the tumor.

This invention is further directed to an immunogenic cellular composition, including a pharmaceutical composition thereof, comprising DCs which have been modified by:

(a) loading the DCs with an antigen so that the antigen is expressed on the DC surface, or transducing or transfecting the DCs with DNA that encodes an antigen fused to an EPP; and

(b) transfecting the DCs with a nucleic acid molecule that is (i) a siNA or (ii) DNA that encodes the siNA, preferably siRNA, wherein the siNA has a sequence that is sufficiently complementary to the sequence of, and thus targets, mRNA that encodes a pro-apoptotic protein expressed in the DC, such that expression or activity the siNA in the cell results in diminution or loss of expression of the mRNA, resulting in inhibition of apoptosis and prolonged survival of the DC.

The pro-apoptotic protein target is preferably one or more of (a) Bak, (b) Bax, (c) caspase-8, (d) caspase-9 and (e) caspase-3. Preferably, th siRNA targets Bak and/or Bax; preferred targeted sequences are SEQ ID NO:4 of Bak and SEQ ID NO:8 of Bax. Preferred siRNA is selected is (a) SEQ ID NO:1/SEQ ID NO:2; or b) SEQ ID NO:5/SEQ ID NO:6.

A method of inducing or enhancing an antigen specific immune response in a subject comprises administering to the subject an effective amount of the above DC composition thereby inducing or enhancing the antigen specific immune response.

A method of increasing the numbers of $CD8^+$ CTLs specific for a selected desired antigen in a subject comprises administering an effective amount of the DC composition wherein the loaded antigen or the antigen expressed from the transduced DNA comprises an epitope that binds to and is presented on the DC surface by MHC class I proteins, thereby increasing the numbers of antigen-specific $CD8^+$ CTLs.

A method of inhibiting the tumor growth in a subject comprises administering an effective amount of the DC composition, wherein the antigenic epitopes are those expressed by the tumor or ones cross reactive with those expressed by the tumor, thereby inhibiting growth of the tumor.

The invention is directed to use of a nucleic acid composition as defined above or particles as defined above or a DC composition as defined above in the manufacture of a medicament, preferably a vaccine, for inducing or enhancing an antigen specific immune response in a subject.

Also included is the use of a nucleic acid composition as defined above, particles as defined above, or a DC composition as defined above in the manufacture of a medicament for inhibiting the growth of a tumor or treating cancer in a subject wherein the antigenic epitopes are those expressed by the tumor or ones cross-reactive with those expressed by the tumor.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1-3. Detection of Bak and Bax expression and evaluation of resistance to apoptotic cell death after delivery **of Bak and/or Bax siRNA.** Fig. 1 is a Western blot analysis demonstrating expression of Bak and/or Bax protein in transfected cells. Fig. 2 is a Western blot demonstrating kinetics of Bak and Bax expression in siRNA-transfected DC-I cells. β-actin was used as an internal control for quantification of protein expression. Fig. 3A-B is a graph showing the percentage of apoptotic cells in E7 peptide-pulsed DC1 cells (RAHYNIVTF, SEQ ID NO:46) transfected with either Bak+Bax siRNA or with control siRNA, after incubation for 4 hrs (Fig. 3A) or 20 hrs (Fig. 3B)) with an E7-specific $CD8^+$ T cell line. DC-I cells pulsed with HA peptide (IYSTVASSL, SEQ ID NO:47) was used as a control.

Figures 4-7. ICCS **and flow cytometric analysis to determine the antigen-specific** $CD8^+$ **T cell response to an immunogenic DNA vaccine coadministered with control or Bax and/or Bak siRNA.** Figs 4 and 5 show results of mice vaccinated with pcDNA3-E7. Figs 6 and 7 shows results of mice vaccinated with pcDNA3-HA or -OVA. pcDNA3 encoding Bak+Bax siRNA served as a negative control. Fig. 4 shows representative flow cytometry results. Fig. 5 represents the number of IFN-γ-expressing E7-specific $CD8^+$ T cells in a population of spleen cells from vaccinated mice. Fig. 6 shows representative flow cytometric data. Fig. 7 represents the number of IFN-γ-expressing HA- or OVA-specific $CD8^+$ T cells in a spleen cell population from vaccinated mice.

Figures 8-10. ***In vivo*** **tumor protection and** therapy **studies using E7-expressing** TC-I **Tumor cells.** Fig. 8 shows results of an *in vivo* tumor protection experiment. pcDNA3 encoding Bak+Bax siRNA was used as a negative control. Fig. 9 shows results of an *in vivo* antibody depletion experiments indicating the contribution of lymphocyte subsets to the observed protective effect above. Fig. 10 shows results of an *in vivo* tumor therapy experiment using the hematogenous spread lung metastasis model. pcDNA3 encoding Bak+Bax siRNA was used as a negative control.

Figures 11-14. ICCS and flow cytometric analysis of E7-specific $CD4^+$ or $CD8^+$ T cell responses in mice vaccinated with a DNA vaccine employing intracellular targeting strategies and siRNA. In the experiments of Figs. 11 and 12, mice were vaccinated with pcDNA3-E7, pcDNA3-Sig/E7/LAMP-1, pcDNA3-E7/HSP70, or pcDNA3-CRT/E7 combined with Bak+Bax siRNA or control siRNA. In the experiments of Figs. 13 and 14, mice were vaccinated with pcDNA3-Sig/E7/LAMP-1 combined with Bak+Bax siRNA or control siRNA. Fig. 13 is a representative example of a flow cytometric analysis. Fig. 12, represents the number of IFN-γ-expressing E7-specific $CD8^+$ T cells in a spleen cell population from vaccinated mice. Fig. 13 represents the number of IFN-γ-expressing E7-specific $CD4^+$ ThI cells in a spleen cell population from vaccinated mice. Fig. 14 represents the number of IL-4-expressing E7-specific $CD4^+$ Th2 cells in the spleen cell population from vaccinated mice.

Figures 15-18. Flow cytometric **analysis of GFP-expressing** DCs **in draining** lymph **nodes of mice vaccinated with E7/GFP DNA combined with Bak+Bax siRNA.** Fig. 15, is a representative sample of a flow cytometry analysis 2 and 5 days after intradermal administration of pcDNA3-E7/GFP. The numbers indicate the percentage of GFP-expressing cells out of the total of CDl lc+ cells. Fig. 16 shows percentages of GFP-expressing cells out of the total of CDl lc+ cells. Fig. 17 shows results of a representative *in vivo* antibody depletion experiment. Fig. 18 shows percentages of GFP+ cells (out of total CDl lc+ cells) after antibody depletion.

Figures 19-20. ICCS **with flow** cytometric analysis **to determine the effect of co¬ administration of Bak+Bax siRNA during priming and/or boosting.** Mice were vaccinated with pcDNA3-Sig/E7/LAMP-1 combined with Bak+Bax siRNA ("B+B") and/or control siRNA ("Con") in the priming phase and/or the boosting phase of the vaccination protocol. Fig. 19 shows representative flow cytometric results. Fig. 20 shows the numbers of IFN-γ-expressing E7-specific $CD8^+$ T cells in the spleen cell population from vaccinated mice.

Figure 21. Western blot analysis **of the expression of Bak and Bax protein in** DC-I **cells transfected with various siRNA constructs.** DC-I cells were transfected with either Bak/Bax siRNA or control siRNA. Western blot analysis was performed with 50μg of cell lysates 24 and 48 hours after transfection. β-actin was used as a control for loading. Lysates of untransfected DC-I cells were used as negative controls.

Figures 22-23. ICCS **and flow** cytometric **analysis to determine the number of IFN-γ-producing E7-specific** $CD8^+$ **T cells in mice after immunization with E7 peptide-pulsed** DCs **transfected with various siRNA constructs.** Mice (5/group) were vaccinated with E7 peptide-loaded DCs transfected with (i) Bak/Bax siRNA or (ii) control siRNA. Mice vaccinated with E7 peptide-loaded DCs (no transfection) were additional controls. Fig. 22 shows representative flow cytometric results for

pooled spleen cells harvested from vaccinated mice that were either (i) stimulated with E7 aa49-57 peptide or (ii) unstimulated in culture. Fig 23 shows the numbers of IFN-γ-secreting E7-specific $CD8^+$ T cell precursors (per 3 X 10^5 spleen cells) from mice vaccinated with E7 peptide-loaded DCs that were transfected with (i) control siRNA, (i) Bak/Bax siRNA or (iii) untransfected. Results shown are means ± SD; /?<0.001; Student's t test).

Figures 24-25. Flow cytometric **analysis (Fig. 24) and ICCS (Fig. 25) of spleen cells from mice immunized with E7-pulsed bone marrow-derived** DCs (BM-DCs) **transfected with the various siRNA constructs.** Mice (5/group) were vaccinated with E7 peptide-loaded BM-DCs transfected with (i) Bak/Bax siRNA or (ii) control siRNA. Fig. 25 represents the number of IFN-γ-secreting E7-specific $CD8^+$ T cell precursors(per 3 x 10^5 spleen cells) after immunization with E7 peptide-loaded BM-DCs transfected with siRNA or from non-immunized mice (mean ± SD; £«0.001; Student's t-test).

Figures 26-27. ***In vivo*** **tumor protection and treatment experiments.** Fig. 26 shows results of a tumor protection experiment in which mice (5/group) were immunized with E7 peptide-loaded DCs transfected with either (i) control siRNA or (ii) Bak/Bax siRNA and boosted after 1 week. 7 days after the last immunization, each mouse was challenged with $5x10^4$ TC-I tumor cells (see Example I). Tumors were monitored twice a week. Unvaccinated mice served as negative controls. Fig. 27 shows results of an *in vivo* tumor therapy experiment in which mice were given E7 peptide-loaded DCs transfected with (i) Bak/Bax siRNA or (ii) control siRNA, three days after TC-I tumor cell challenge ($5x10^4$ tumor cells). Mice were boosted with the same dose and regimen of E7 peptide-loaded DCs one week later and sacrificed 28 days after tumor challenge. Lung nodules (experimental metastases) were evaluated and the results expressed as the mean number of lung nodules ± SD (p<0.001; Student's t-test).

Figures 28A-28B. Survival of E7 peptide-loaded BM-DCs **transfected with Bak/Bax siRNA or control siRNA after administration of E7-specific** $CD8^+$ **T cells** ***in vivo.*** Fig. 28A presents flow cytometric results showing the different level of carboxyfluorescein (CFSE) -labeled E7 peptide-loaded BM-DCs transfected with either (i) Bak/Bax siRNA ("low CFSE") or (ii) control siRNA ("high CFSE"). Bak/Bax-transfected BM-DCs were labeled with a lower concentration (0.5μM) of CFSE, whereas control BM-DCs (transfected with control siRNA) were labeled with a higher concentration (5μM) of CFSE. A representative graph shows the presence of similar numbers of "low CFSE"- labeled E7 peptide-loaded BM-DCs transfected with Bak/Bax siRNA and "high CFSF'-labeled E7 peptide-loaded control BM-DCs before i.v. injection. Fig. 28B shows flow cytometric results demonstrating the ratio of "low CFSE" to "high CFSE" E7 peptide-loaded BM-DCs that have localized to the spleen and

lungs of mice 16 hrs after i.v. injection of a mixture equal numbers (2.5xl0 ^{5}/mouse) of "low CFSE" E7 peptide-loaded BM-DCs transfected with Bak/Bax siRNA and "high CFSE" E7 peptide-loaded control BM-DCs. These CFSE-labeled BM-DCs were injected into mice 3 days after the administration of 10^6 E7-specific T cells/mouse. Contact with these T cells are the basis for DC apoptosis in this study. Note that the number of "low CFSE" cells was significantly higher than the number of "high CFSE" cells.

Figure 29. Characterization of the surface molecules of E7 peptide-loaded DCs after transfection with Bak/Bax siRNA or control siRNA. Flow cytometry was used to determine the level of expression of CDl Ic, CD40, CD86, MHC I, and MHC II-molecules in E7 peptide-loaded murine DC-1 cells transfected with either (i) Bak/Bax siRNA or (ii) control siRNA. E7 peptide-loaded DCs that were not transfected at all served as negative controls.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to one of two fundamental approaches to the improvement of molecular vaccine potency. As the present inventors discovered, in addition to DNA encoding an antigen, the concomitant administration of a second DNA molecule encoding an siRNA specific for an apoptotic protein, preferably Bak and/or Bax, termed "anti-apoptotic siRNA DNA" for simplicity), enhances the magnitude and/or duration of a T cell mediated immune response, and potentiates a desired clinical effect - such as eradication of an existing tumor or prevention of the spread or metastasis of a tumor.

The anti-apoptotic siRNA DNA may be physically linked to the antigen-encoding DNA. Alternatively, and preferably, the anti-apoptotic siRNA DNA may be administered separately from, but in combination with the antigen-encoding DNA molecule. Examples of the co-administration of these two types of vectors is provided.

This strategy may be combined with an additional strategy pioneered by the present inventors and colleagues, that involve linking DNA encoding another protein, generically termed a "zmmunogenicity-p_otentiating polypeptide" or "IPP" to the antigen-encoding DNA. Again, for the sake of simplicity, the DNA encoding such a targeting polypeptide will be referred to herein as a "IPP DNA." That strategy has been shown to be effective in enhancing the potency of the vectors carrying only antigen-encoding DNA. See for example: Wu *et al,* WO 01/29233; Wu *et al,* WO 02/009645; Wu *et al,* WO 02/061113; Wu *etal,* WO 02/074920; Wu *et al,* WO 02/12281, all of which are incorporated by reference in their entirety.

The details of the various targeting polypeptide strategies will not be discussed in detail herein, although several such vectors are useful in the present invention and their sequences are provided below. The preferred IPPs include Sig/LAMP-1, the translocation domain, which is domain π (dll) of

Pseudomonas aeruginosa exotoxin A (ETA(dII)) or from similar toxins from *Diptheria, Clostridium, Botulinum, Bacillus, Yersinia, Vibrio cholerae,* or *Bordetella),* an endoplasmic reticulum chaperone polypeptide exemplified by calreticulin (CRT) but also including ER60, GRP94 or gp96, well-characterized ER chaperone polypeptide that representatives of the HSP90 family of stress-induced proteins (see WO 02/012281), VP22 protein from herpes simplex virus and its homologues from other herpes viruses such as Marek's disease virus (see WO 02/09645), mycobacterial heat shock protein HSP70 (WO0129233, U.S. Patent 6,734,173; WO02061 113) , and γ-tubulin (Hung CF *et al, Cane Res 63:2393-98,* 2003)

DNA encoding each of these polypeptides, or fragments or variants thereof with substantially the same biological activity, when linked to an antigen-encoding or epitope-encoding DNA molecule, result in more potent T cell mediate responses to the antigen compared to immunization with the antigen-encoding DNA alone. These polypeptide can be considered as "molecular adjuvants." These effects are manifest primarily with CD8+ T cells, although some of these approaches induce potent CD4+ T cell mediated effects as well.

The results presented herein prove that molecular vaccination with

(a) a combination of an antigen-encoding DNA and an anti-apoptotic siRNA DNA; or

(b) a combination of a chimeric DNA encoding (i) antigen plus (ii) an IPP and an anti-apoptotic siRNA

or a combination of (a) and (b) above, will results in a stronger and more durable immune response which can be protective and/or therapeutic. A related embodiment that is expected to give similar results is:

(c) a combination of a chimeric DNA comprising an antigen-encoding DNA sequence optionally linked to an IPP-encoding DNA and a DNA composition encoding an anti-apoptotic siRNA.

Also included within the scope of this invention (compositions and methods for inducing more potent immune responses, is a DC that is (i) loaded with, and therefore presenting, an antigen, and (ii) transfected with siRNA or with DNA encoding siRNA directed to Bak/Bax that suppress or reverse Bak/Bax protein expression. Such transfected DCs are preferably "pulsed" (or "loaded") with an antigenic peptide. The DCs may be transfected *ex vivo* with anti-apoptotic siRNA or siRNA DNA, and loaded with antigen *ex vivo.* Alternatively, the transfection or loading or both may take place *in vivo.* If transfection or loading takes place *ex vivo,* the other may be conducted *in vivo* , either prior to removing the cells for *ex vivo* treatment or after the *ex vivo* treated cells have been administered to the subject.

Any one of the types of vectors may also comprise DNA encoding an immunostimulatory cytokine, preferably those that target APCs, preferably DCs, such as granulocyte macrophage colony stimulating factor (GM-CSF), or active fragments or domains thereof, and/or DNA encoding a

costimulatory signal, such as a B7 family protein, including B7-DC (see US Pat. App. Serial No. 09/794,210), B7.1, B7.2, soluble CD40, *etc.).*

The vectors used to deliver the foregoing DNA sequences include naked DNA vectors (plasmids), DNA-based alphaviral RNA replicons ("suicidal DNA vectors") and self replicating RNA replicons.

The order in which the two (or more) components of a chimeric DNA vaccine construct (antigen and EPP) are arranged, and therefore, the order of the encoding nucleic acid fragments in the nucleic acid vector, can be altered without affecting immunogenicity of the fusion polypeptides proteins and the utility of the composition. As has been disclosed by the present inventors and their colleagues in other published patent applications cited herein, for some combinations of antigen/IPP, one order is preferred, *e.g.* E7. ..HSP70 and CRT. ..E7 (indicating N- to C- terminal in the polypeptide).

The experiments described herein demonstrate that the methods of the invention can enhance a cellular immune response, particularly, tumor-destructive CTL reactivity, induced by a DNA vaccine encoding an epitope of a human pathogen. Human HPV-16 E7 was used as a model antigen for vaccine development because human papillomaviruses (HPVs), particularly HPV-16, are associated with most human cervical cancers. The oncogenic HPV proteins E7 and E6 are important in the induction and maintenance of cellular transformation and co-expressed in most HPV-containing cervical cancers and their precursor lesions. Therefore, cancer vaccines, such as the compositions of the invention, that target E7 can be used to control of HPV-associated neoplasms (Wu (1994) *Curr. Opin. Immunol.* (5:746-754). Similar DNA vaccines encoding E6 are also included herein and studies with E6 using the IPPs disclosed herein have shown successful potentiation of E6-specific immune responses.

Based on studies of simultaneous vaccination with both CRT/E6 and CRT/E7 DNA vaccines generated significant E6- and E7-specific T-cell immune responses and significantly better therapeutic antitumor effects against E6- and E7-expressing tumors than vaccination with either CRT/E6 DNA or CRT/E7 DNA alone.

In one embodiment, the present invention is directed to simultaneous vaccination with both E6 and E7 DNA immunogens, or IPP/E6 and *WVIKl* DNA immunogens, in combination with delivery of siRNA targeting mRNA encoding pro-apoptotic proteins, preferably Bak and/or Bax.

The present invention is not limited to the exemplified antigen(s). Rather, one of skill in the art will appreciate that the same results are expected for any antigen (and epitopes thereof) for which a T cell-mediated response is desired. The response so generated will be effective in providing protective or therapeutic immunity, or both, directed to an organism or disease in which the epitope or antigenic determinant is involved - for example as a cell surface antigen of a pathogenic cell or an envelope or

other antigen of a pathogenic virus, or a bacterial antigen, or an antigen expressed as or as part of a pathogenic molecule.

Thus, in one embodiment, the antigen *(e.g.,* the MHC class I-binding peptide epitope) is one that is derived from a pathogen, *e.g.,* a peptide expressed by a pathogen. The pathogen can be a virus, such as, *e.g., a* papilloma virus, a herpesvirus, a retrovirus (including an immunodeficiency virus, such as HIV-I), an adenovirus, and the like. The papilloma virus can be a human papilloma virus, for which a preferred antigen *(e.g.,* a MHC class I-binding peptide) can be the HPV-16 E6 or E7 polypeptide or an immunogenic fragment thereof. In one embodiment employing E6 and/or E7, the polypeptide is rendered substantially non-oncogenic by about 1 to about 3 amino acid substitutions that maintain immunogenicity while destroying oncogenicity, for example, by destroying the ability of the polypeptide to bind retinoblastoma polypeptide (pRB) or substantially lowering the affinity for pRB. As a result, the E7 polypeptide is effectively non-oncogenic when expressed *in vivo* or delivered *in vivo.*

In alternative embodiments, the pathogen is a bacteria, such as *Bordetell αpertussis; Ehrlichia chaffeensis; Staphylococcus aureus; Toxoplasma gondii; Legionella pneumophila; Brucella suis; Salmonella enterica; Mycobacterium avium; Mycobacterium tuberculosis; Listeria monocytogenes; Chlamydia trachomatis; Chlamydia pneumoniae; Rickettsia rickettsii;* or, a fungus, such as, *e.g., Paracoccidioides brasiliensis;* or other pathogen, *e.g., Plasmodium falciparum.*

In another embodiment, the MHC class I-binding peptide epitope is derived from a tumor cell. The tumor cell-derived peptide epitope can comprise a tumor associated antigen, e.g., a tumor specific antigen, such as, *e.g.,* a HER-2/neu antigen, or one of a number of known melanoma antigens, *etc.*

In one embodiment, the isolated or recombinant nucleic acid molecule is operatively linked to a promoter, such as, *e.g.,* a constitutive, an inducible or a tissue-specific promoter. The promoter can be expressed in any cell, including cells of the immune system, including, *e.g.,* antigen presenting cells (APCs), *e.g.,* in a constitutive, an inducible or a tissue-specific manner.

In alternative embodiments, the APCs are DCs, keratinocytes, astrocytes, monocytes, macrophages, B lymphocytes, a microglial cell, or activated endothelial cells, and the like.

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art of this invention. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The term "antigen" or "immunogen" as used herein refers to a compound or composition or cell comprising a peptide, polypeptide or protein which is "antigenic" or "immunogenic" when administered in an appropriate amount (an "immunogenically effective amount"), *Le.,* capable of inducing, eliciting, augmenting or boosting a cellular and/or humoral immune response and of being recognized by the products of that response (T cells, antibodies). A nucleic acid such as DNA that encodeds an

immunogen and is used as a vaccine is referred to as a "DNA immunogen" as the encoded polypeptide is expressed *in vivo* after administration of the DNA. An immunogen may be effective when given alone or in combination, or linked to, or fused to, another substance (which can be administered at one time or over several intervals). An immunogenic composition can comprise an antigenic peptide/polypeptide of at least about 5, or about 10 or about 15, or about 20 amino acids, *etc.* Smaller antigens may require presence of a "carrier" polypeptide *e.g.,* as a fusion protein, aggregate, conjugate or mixture, preferably linked (chemically or otherwise) to the antigen to be immunogenic. The immunogen can be recombinantly expressed from a vaccine vector, which can be naked DNA which comprises the polypeptide immunogen's coding sequence operably linked to a promoter, *e.g.,* an expression vector or cassette as described herein. The immunogen includes one or more antigenic determinants or epitopes which may vary in size from about 3 to about 15 amino acids.

The term "epitope" as used herein refers to an antigenic determinant or antigenic site that interacts with an antibody or a T cell receptor (TCR), *e.g.,* the MHC class I-binding peptide compositions (or expressed products of the nucleic acid compositions of the invention) used in the methods of the invention. An "antigen" is a molecule or chemical structure that either induces an immune response or is specifically recognized or bound by the product or mediator of an immune response, such as an antibody or a CTL. The specific conformational or stereochemical "domain" to which an antibody or a TCR bind is an "antigenic determinant" or "epitope." TCRs bind to peptide epitopes which are physically associated with a third molecule, a major histocompatibility complex (MHC) class I or class π protein.

The term "recombinant" refers to (1) a nucleic acid or polynucleotide synthesized or otherwise manipulated *in vitro,* (2) methods of using recombinant DNA technology to produce gene products in cells or other biological systems, or (3) a polypeptide encoded by a recombinant nucleic acid. For example, the ETA(dü)-encoding nucleic acid or polypeptide, the nucleic acid encoding an MHC class I-binding peptide epitope (antigen) or the peptide itself can be recombinant. "Recombinant means" includes ligation of nucleic acids having various coding regions or domains or promoter sequences from different sources into a single unit in the form of an expression cassette or vector for expression of the coding sequences in the vectors resulting in production of the encoded polypeptide.

The term "self-replicating RNA replicon" refers to a construct based on an RNA viruses, such as alphavirus genome RNAs *(e.g.,* Sindbis virus, Semliki Forest virus, *etc.)* that have been engineered to allow expression of heterologous RNAs and proteins. These recombinant vectors are self-replicating ("replicons") which can be introduced into cells as naked RNA or DNA, as described in detail in co-

pending, commonly assigned U.S. and PCT patent applications by the present inventors (USSN 10/060,274 and WO 02/061 113).

siRNAs

The present inventors designed siRNA sequences that hybridize to, and block expression of th activation of Bak and Bax proteins that are central players in the apoptosis signalling pathway. The present invention is directed to the siRNA molecules (sequences), vectors containing or encoding the siRNA, expression vectors with a promoter operably linked to the siRNA coding sequence that drives transcription of siRNA sequences that are "specific" for sequences Bak and Bax nucleic acid. siRNAs may include single stranded "hairpin" sequences because of their stability and binding to the target mRNA.

Since Bak and Bax are involved, among other death proteins, in apoptosis of APCs, particularly DCs, the present siRNA sequences may be used in conjunction with a broad range of DNA vaccine constructs encoding antigens to enhance and promote the immune response induced by such DNA vaccine constructs, particularly CD8+ T cell mediated immune responses typified by CTL activation and action. This is believed to occur as a result of the effect of the siRNA in prolonging the life of antigen-presenting DCs which may otherwise be killed in the course of a developing immune response by the very same CTLs that the DCs are responsible for inducing.

In addition to Bak and Bax, additional targets for siRNAs designed in an analogous manner include caspase 8, caspase 9 and caspase 3. These proteins and their role in apoptosis was described above. The present invention includes compositions and methods in which siRNAs targeting any two or more of Bak, Bax, caspase 8, caspase 9 and caspase 3 are used in combination, optionally simultaneously (along with a DNA immunogen that encodes an antigen), to administer to a subject. Such combinations of siRNAs may also be used to transfect DCs (along with antigen loading) to improve the immunogenicity of the DCs as cellular vaccines by rendering them resistant to apoptosis.

siRNAs suppress gene expression through a highly regulated enzyme-mediated process called RNA interference (RNAi) (Sharp, P.A., *Genes Dev.* 15:485-90, 2001; Bernstein, E *et al., Nature* 409:363-66, 2001; Nykanen, A *et al, Cell* 107:309-21, 2001; Elbashir *et al, Genes Dev.* 15:188-200, 2001). RNA interference is the sequence-specific degradation of homologues in an mRNA of a targeting sequence in an siNA. As used herein, the term siNA (small, or short, interfering nucleic acid) is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi (RNA interference), for example short (or small) interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-

modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), translational silencing, and others. RNAi involves multiple RNA-protein interactions characterized by four major steps: assembly of siRNA with the RNA-induced silencing complex (RISC), activation of the RISC, target recognition and target cleavage. These interactions may bias strand selection during siRNA-RISC assembly and activation, and contribute to the overall efficiency of RNAi (Khvorova, A *et al.*, *Cell* 115:209-216 (2003); Schwarz, DS *etal.* 115:199-208 (2003)))

Considerations to be taken into account when designing an RNAi molecule include, among others, the sequence to be targeted, secondary structure of the RNA target and binding of RNA binding proteins. Methods of optimizing siRNA sequences will be evident to the skilled worker. Typical algorithms and methods are described in Vickers *et al.* (2003) *J Biol Chem* 275:7108-71 18; Yang *et al.* (2003) *Proc Natl Acad Sci USA* 99:9942-9947; Far *et al.* (2003) *Nuc. Acids Res.* 3i:4417-4424; and Reynolds *et al.* (2004) *Nature Biotechnology* 22:326-330, all of which are incorporated by reference in their entirety.

The methods described in Far *et al, supra,* and Reynolds *et al, supra,* may be used by those of ordinary skill in the art to select targeted sequences and design siRNA sequences that are effective at silencing the transcription of the relevant mRNA. Far *et al* suggests options for assessing target accessibility for siRNA and supports the design of active siRNA constructs. This approach can be automated, adapted to high throughput and is open to include additional parameters relevant to the biological activity of siRNA. To identify siRNA-specific features likely to contribute to efficient processing at each of the steps of RNAi noted above. Reynolds *et al, supra,* present a systematic analysis of 180 siRNAs targeting the mRNA of two genes. Eight characteristics associated with siRNA functionality were identified: low G/C content, a bias towards low internal stability at the sense strand 3'-terminus, lack of inverted repeats, and sense strand base preferences (positions 3, 10, 13 and 19). Application of an algorithm incorporating all eight criteria significantly improves potent siRNA selection. This highlights the utility of rational design for selecting potent siRNAs that facilitate functional gene knockdown.

Candidate siRNA sequences against mouse and human Bax and Bak are selected using a process that involves running a BLAST search against the sequence of Bax or Bak (or any other target) and selecting sequences that "survive" to ensure that these sequences will not be cross matched with any other genes.

siRNA sequences selected according to such a process and algorithm may be cloned into an expression plasmid and tested for their activity in abrogating Bak/Bax function cells of the appropriate animal species. Those sequences that show RNAi activity may be used by direct administration bound

to particles, or recloned into a viral vector such as a replication-defective human adenovirus serotype 5 (Ad5).

One advantage of this viral vector is the high titer obtainable (in the range of 10^{10}) and therefore the high multiplicities-of infection that can be attained. For example, infection with 100 infectious units/ cell ensures all cells are infected. Another advantage of this virus is the high susceptibility and infectivity and the host range (with respect to cell types). Even if expression is transient, cells would survive, possibly replicate, and continue to function before Bak/Bax activity would recover and lead to cell death. Preferred constructs described in the Examples are the following:

For **Bak:**

5 ' P-UGCCUACGAACUCUUCACCCITdT-S' (sense) (SEQ ID NO:1)
5 ' P-GGUGAAGAGUUCGUAGGCAdTdT-3 ' (antisense) (SEQ ID NO:2),

The nucleotide sequence encoding the Bak protein (including the stop codon) (GenBank accession No. NM_007523 is shown below (SEQ ID NO:3) with the targeted sequence in upper case, underscored.

atggcatctggacaaggaccaggtcccccgaaggtgggctgcgatgagtccccgtccccttctgaacagc
aggttgcccaggacacagaggaggtctttcgaagctacgtttttttacctccaccagcaggaacaggagac
ccaggggcggccgcctgccaaccccgagatggacaacttgcccctggaacccaacagcatcttgggtcag
gtgggtcggcagcttgctctcatcggagatgatattaaccggcgctacgacacagagttccagaatttac
taqaacaqcttcaqcccacaqccqqqaa TGCCTACGAACTCTTCACC aaqatcqcctccaqcctatttaa
gagtggcatcagctggggccgcgtggtggctctcctgggctttggctaccgtctggccctgtacgtctac
cagcgtggtttgaccggcttcctgggccaggtgacctgctttttggctgatatcatactgcatcattaca
tcgccagatggatcgcacagagaggcggttgggtggcagccctgaatttgcgtagagaccccatcctgac
cgtaatggtgatttttggtgtggttctgttgggccaattcgtggtacacagattcttcagatcatga 637

The targeted sequence of Bak, TGCCTACGAACTCTTCACC is SEQ ID NO:4

For Bax:

5 'P-UAUGGAGCUGCAGAGGAUGdTdT- 3 ' (sense) (SEQ ID NO:5)
5 'P-CAUCCUCUGCAGCUCCAUAdTdT- 3 ' (antisense) (SEQIDNO:6)

The nucleotide sequence encoding Bax (including the stop codon) (GenBank accession No. L22472 is shown below (SEQ ID NO:7) with the targeted sequence shown in upper case and underscored

atggacgggtccggggagcagcttgggagcggcgggcccaccagctctgaacagatcatgaagacagggg
ccttttttgctacagggtttcatccaggatcgagcagggaggatggctggggagacacctgagctgacctt
ggagcagccgccccaggatgcgtccaccaagaagctgagcgagtgtctccggcgaattggagatgaactg
gataqca aTATGGAGCTGCAGAGGATG attqctqacqtqqacacqqacqcccccccqaqaqqtcttcttcc
gggtggcagctgacatgtttgctgatggcaacttcaactggggccgcgtggttgccctcttctactttgc
tagcaaactggtgctcaaggccctgtgcactaaagtgcccgagctgatcagaaccatcatgggctggaca
ctggacttcctccgtgagcggctgcttgtctggatccaagaccagggtggctgggaaggcctcctctcct
acttcgggacccccacatggcagacagtgaccatctttgtggctggagtcctcaccgcctcgctcaccat
ctggaagaagatgggctga 589

The targeted sequence of Bax, TATGGAGCTGCAGAGGATG is SEQ ID NO: 8

In a preferred embodiment, the inhibitory molecule is a double stranded nucleic acid (preferably an RNA), used in a method of RNA interference. The following show the "paired" 19 nucleotide structures of the siRNA sequences shown above, where the symbol *t* :

Bak: 5 ' P- UGCCUACGAACUCUUCACCdTdT-3 ' (sense) (SEQ ID NO:1)

tπm πππππππm

3 ' P-dTdtACGGAUGCUUGAGAAGUGG - 5 ' (antisense) (SEQ ID NO:2)

Bax: 5 ' P- UAUGGAGCUGCAGAGGAUGdTdT- 3 ' (sense) (SEQ ID NO:5)

πmtmm πm π

3 ' P-dTdTAUACCUCGACGUCUCCUAC - 5 ' (antisense) (SEQ ID NO:6)

Other Pro-Apoptotic Proteins to be Targeted

1. Caspase 8: The nucleotide sequence of human caspase-8 is shown below (SEQ ID NO:9). GenBank Access. # NM_001228. One target sequence for RNAi is underscored. Others may be identified using methods such as those described herein (and in reference cited herein, primarily Far *et al, supra* and Reynolds *et ah, supra).*

```
atg gac ttc age aga aat ctt tat gat att ggg gaa caa ctg gac agt gaa gat ctg gcc
tec etc aag ttc ctg age ctg gac tac att ccg caa agg aag caa gaa ccc ate aag gat
gcc ttg atg tta ttc cag aga etc cag gaa aag aga atg ttg gag gaa age aat ctg tec
ttc ctg aag gag ctg etc ttc cga att aat aga ctg gat ttg ctg att ace tac eta aac
act aga aag gag gag atg gaa agg gaa ctt cag aca cca ggc agg get caa att tct gcc
tac agg ttc cac ttc tgc cgc atg age tgg get gaa gca aac age cag tgc cag aca cag
tct gta cct ttc tgg egg agg gtc gat cat eta tta ata agg gtc atg etc tat cag att
tea gaa gaa gtg age aga tea gaa ttg agg tct ttt aag ttt ctt ttg caa gag gaa ate
tec aaa tgc aaa ctg gat gat gac atg aac ctg ctg gat att ttc ata gag atg gag aag
agg gtc ate ctg gga gaa gga aag ttg gac ate ctg aaa aga gtc tgt gcc caa ate aac
aag age ctg ctg aag ata ate aac gac tat gaa gaa ttc age aaa ggg gag gag ttg tgt
ggg gta atg aca ate teg gac tct cca aga gaa cag gat agt gaa tea cag act ttg gac
aaa gtt tac caa atg aaa age aaa cct cgg gga tac tgt ctg ate ate aac aat cac aat
ttt gca aaa gca egg gag aaa gtg ccc aaa ctt cac age att agg gac agg aat gga aca
cac ttg gat gca ggg get ttg ace acg ace ttt gaa gag ctt cat ttt gag ate aag ccc
cac gat gac tgc aca gta gag caa ate tat gag att ttg aaa ate tac caa etc atg gac
cac agt aac atg gac tgc ttc ate tgc tgt ate etc tec cat gga gac aag ggc ate ate
tat ggc act gat gga cag gag gcc ccc ate tat gag ctg aca tct cag ttc act ggt ttg
aag tgc cct tec ctt get gga aaa ccc aaa gtg ttt ttt att cag get tgt cag ggg gat
aac tac cag aaa ggt ata cct gtt gag act gat tea gag gag caa ccc tat tta gaa atg
gat tta tea tea cct caa acg aga tat ate ccg gat gag get gac ttt ctg ctg ggg atg
gcc act gtg aat aac tgt gtt tec tac cga aac cct gca gag gga ace tgg tac ate cag
tea ctt tgc cag age ctg aga gag cga tgt cct cga ggc gat gat att etc ace ate ctg
act gaa gtg aac tat gaa gta age aac aag gat gac aag aaa aac atg ggg aaa cag atg
cct cag cct act ttc aca eta aga aaa aaa ctt gtc ttc cct tct gat tga 1491
```

The sequences of sense and antisense siRNA strands for targeting this sequence (including dTdT 3 ' overhangs, are:

5 '-AACCUCGGGGAUACUGUCUGAdTdT- 3 ' (sense) (SEQ ID NO: 10)

5 '-UCAGACAGUAUCCCCGAGGUUdTdT- 3 ' (antisense)

2. Caspase 9: The nucleotide sequence of human caspase-9 is shown below (SEQ ID NO: 12). See GenBank Access. # NM_001229. The sequence below is of "variant α" which is longer than a second alternatively spliced variant β, which lacks the underscored part of the sequence shown below (and

which is anti-apoptotic). Target sequences for RNAi, expected to fall in the underscored segment, are identified using known methods such as those described herein and in Far *et at, supra* and Reynolds *et ah, supra).* and siNAs, such as siRNAs, are designed accordingly.

atg gac gaa gcg gat egg egg etc ctg egg egg tgc egg ctg egg ctg gtg gaa gag ctg
cag gtg gac cag etc tgg gac gcc ctg ctg age cgc gag ctg ttc agg ccc cat atg ate
gag gac ate cag egg gca ggc tct gga tct egg egg gat cag gcc agg cag ctg ate ata
gat ctg gag act cga ggg agt cag get ctt cct ttg ttc ate tec tgc tta gag gac aca
ggc cag gac atg ctg get teg ttt ctg cga act aac agg caa gca gca aag ttg teg aag
cca ace eta gaa aac ctt ace cca gtg gtg etc aga cca gag att cgc aaa cca gag gtt
etc aga ccg gaa aca ccc aga cca gtg gac att ggt tct gga gga ttt ggt gat gtc qqt
qct ctt qag agt ttq agg qga aat gca gat ttq qct tac ate ctq agc atg qag ccc tqt
qqc cac tqc etc att ate aac aat qtq aac ttc tqc cqt gag tec qqq etc cgc ace cqc
act qqc tec aac ate gac tqt qaq aag ttq cgg cqt cgc ttc tec tcq ctq cat ttc atg
qtq qaq qtq aag qqc gac ctq act qcc aag aaa atg qtq ctq qct ttq ctq gag ctq qcq
caq cag gac cac qqt qct ctq gac tqc tqc gtg qtq qtc att etc tct cac qqc tqt caq
qcc agc cac ctq cag ttc cca ggg qct qtc tac ggc aca gat gga tqc CCt gtg tcq qtc
qaq aag att qtq aac ate ttc aat gg q ace agc tqc ccc agc ctq qqa ggg aag ccc aag
etc ttt ttc ate caq qcc tqt qqt qqq gag cag aaa gac cat ggg ttt gag gtg gcc tec
act tec cct gaa gac gag tec cct ggc agt aac ccc gag cca gat gcc ace ccg ttc cag
gaa ggt ttg agg ace ttc gac cag ctg gac gcc ata tct agt ttg ccc aca ccc agt gac
ate ttt gtg tec tac tct act ttc cca ggt ttt gtt tec tgg agg gac ccc aag agt ggc
tec tgg tac gtt gag ace ctg gac gac ate ttt gag cag tgg get cac tct gaa gac ctg
cag tec etc ctg ctt agg gtc get aat get gtt teg gtg aaa ggg att tat aaa cag atg
cct ggt tgc ttt aat ttc etc egg aaa aaa ctt ttc ttt aaa aca tea *taa* 1191

3. Caspase 3: The nucleotide sequence of human caspase-3 is shown below (SEQ ID NO: 13). See GenBank Access. # NM_004346. The sequence below is of "variant oc" which is the longer of two alternatively spliced variants, all of which encode the full protein. Target sequences for RNAi are identified using known methods such as those described herein and in Far *et at, supra* and Reynolds *et ah, supra)* and siNAs, such as siRNAs, are designed accordingly.

atg gag aac act gaa aac tea gtg gat tea aaa tec att aaa aat ttg gaa cca aag ate
ata cat gga age gaa tea atg gac tct gga ata tec ctg gac aac agt tat aaa atg gat
tat cct gag atg ggt tta tgt ata ata att aat aat aag aat ttt cat aaa age act gga
atg aca tct egg tct ggt aca gat gtc gat gca gca aac etc agg gaa aca ttc aga aac
ttg aaa tat gaa gtc agg aat aaa aat gat ctt aca cgt gaa gaa att gtg gaa ttg atg
cgt gat gtt tct aaa gaa gat cac age aaa agg age agt ttt gtt tgt gtg ctt ctg age
cat ggt gaa gaa gga ata att ttt gga aca aat gga cct gtt gac ctg aaa aaa ata aca
aac ttt ttc aga ggg gat cgt tgt aga agt eta act gga aaa ccc aaa ctt ttc att att
cag gcc tgc cgt ggt aca gaa ctg gac tgt ggc att gag aca gac agt ggt gtt gat gat
gac atg gcg tgt cat aaa ata cca gtg gag gcc gac ttc ttg tat gca tac tec aca gca
cct ggt tat tat tct tgg cga aat tea aag gat ggc tec tgg ttc ate cag teg ctt tgt
gcc atg ctg aaa cag tat gcc gac aag ctt gaa ttt atg cac att ctt ace egg gtt aac
cga aag gtg gca aca gaa ttt gag tec ttt tec ttt gac get act ttt cat gca aag aaa
cag att cca tgt att gtt tec atg etc aca aaa gaa etc tat ttt tat cac taa 834

Long double stranded interfering RNAs, such a miRNAs, appear to tolerate mismatches more readily than do short double stranded RNAs. In addition, as used herein, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, or an epigenetic phenomenon. For example, siNA molecules of the invention can be used to epigenetically silence genes at both the post-transcriptional level or the pre-transcriptional level. In a non-limiting example, epigenetic regulation of gene expression by siNA molecules of the invention can result from siNA mediated modification of chromatin structure and

thereby alter gene expression (see, for example, Allshire *Science* 297:1818-19, 2002; Volpe *et al., Science 297:1833-37* , 2002; Jenuwein, *Science* 297:2215-18, 2002; and Hall *et al., Science 297,* 2232-2237, 2002.)

An siNA can be designed to target any region of the coding or non-coding sequence of an mRNA. An siNA is a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region has a nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary. The siNA can be assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the siNA are linked by means of a nucleic acid based or non-nucleic acid-based linker(s). The siNA can be a polynucleotide with a hairpin secondary structure, having self-complementary sense and antisense regions. The siNA can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the circular polynucleotide can be processed either *in vivo* or *in vitro* to generate an active siNA molecule capable of mediating RNAi. The siNA can also comprise a single stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (or can be an siNA molecule that does not require the presence within the siNA molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate (see for example Martinez *et al.* (2002) *Cell 110,* 563-574 and Schwarz *et al.* (2002) *Molecular Cell 10,* 537-568), or 5',3'-diphosphate.

In certain embodiments, the siNA molecule of the invention comprises separate sense and antisense sequences or regions, wherein the sense and antisense regions are covalently linked by nucleotide or non-nucleotide linkers molecules as is known in the art, or are alternately non-covalently linked by ionic interactions, hydrogen bonding, Van der Waal's interactions, hydrophobic interactions, and/or stacking interactions. Some preferred siRNAs are discussed above and in the Examples.

As used herein, siNA molecules need not be limited to those molecules containing only ribonucleotides but may also further encompass deoxyribonucleotides (as in the preferred siRNAs which each include a dTdT dinucleotide) chemically-modified nucleotides, and non-nucleotides. In certain embodiments, the siNA molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides. In certain embodiments, siNAs do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, siNAs of the invention optionally do not include any ribonucleotides *{e.g.,*

nucleotides having a 2'-OH group). Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, siNA molecules can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions. If modified, the siNAs of the invention can also be referred to as "short interfering modified oligonucleotides" or "siMON." Other chemical modifications, *e.g.,* as described in Int'l Patent Publications WO 03/0709 18and WO 03/074654, can be applied to any siNA sequence of the invention.

Preferably a molecule mediating RNAi has a 2 nucleotide 3' overhang (dTdT in the preferred sequences disclosed herein). If the RNAi molecule is expressed in a cell from a construct, for example from a hairpin molecule or from an inverted repeat of the desired sequence, then the endogenous cellular machinery will create the overhangs.

Methods of making siRNAs are conventional. *In vitro* methods include processing the polyribonucleotide sequence in a cell-free system *{e.g.,* digesting long dsRNAs with RNAse in or Dicer), transcribing recombinant double stranded DNA *in vitro,* and, preferably, chemical synthesis of nucleotide sequences homologous to Bak or Bax sequences. See, *e.g.,* Tuschl *et al, Genes & Dev.* 73:3191-3197, 1999. *In vivo* methods include

(1) transfecting DNA vectors into a cell such that a substrate is converted into siRNA *in vivo.* See, for example, Kawasaki *et al., Nucleic Acids Res* 37:700-07, 2003;; Miyagishi *et al, Nature Biotechnol* 20:497-500, 2003;; Lee *et al, Nature Biotechnol* 20:500-05, 2002; Brummelkamp *et al, Science* 296:550-53, 2002; McManus *et al, RNA* 8:842-50, 2002; Paddison *et al, Genes Dev* 7(5:948-58, 2002; Paddison *et al, Proc Natl Acad Sci USA* 99:1443-48, 2002; Paul *et al, Nature Biotechnol* 20:505-08, 2002; Sui *et al, Proc Natl Acad Sci USA* 99:5515-20, 2002; Yu *et al, Proc Natl Acad Sci USA* 99:6047-52, 2002)

(2) expressing short hairpin RNAs from plasmid systems using RNA polymerase HI (pol EI) promoters. See, for example, Kawasaki *et al, supra;* Miyagishi *et al, supra;* Lee *et al, supra;* Brummelkamp *et al, supra;* McManus *et al, supra),* Paddison *et al, supra* (both); Paul *et al, supra,* Sui *et al, supra;* and Yu *et al, supra;* and/or

(3) expressing short RNA from tandem promoters. See, for example, Miyagishi *et al, supra;* Lee *et al, supra).*

When synthesized *in vitro,* a typical micromolar scale RNA synthesis provides about 1 mg of siRNA, which is sufficient for about 1000 transfection experiments using a 24-well tissue culture plate format. In general, to inhibit Bak or Bax expression in cells in culture, one or more siRNAs can be added to cells in culture media, typically at about 1 ng/ml to about 10 μg siRNA/ml.

For reviews and more general description of inhibitory RNAs, see Lau *et al, Sci Amer* Aug 2003: 34-41; McManus *et al, Nature Rev Genetics 3,* 737-47, 2002; and Dykxhoorn *et al, Nature Rev MoI Cell Bio 4:457-461,* 2003. For further guidance regarding methods of designing and preparing siRNAs, testing them for efficacy, and using them in methods of RNA interference (both *in vitro* and *in vivo),* see, *e.g.,* Allshire, *Science* 297:1818-19, 2002; Volpe *et al, Science* 297:1833-37, 2002; Jenuwein, *Science* 297:2215-18, 2002; Hall *et al, Science 297* 2232-37, 2002; Hutvagner *et al, Science* 297:2056-60, 2002; McManus *et al RNA* 5:842-850, 2002; Reinhart *et al, Genes Dev.* id: 1616-26, 2002; Reinhart *et al, Science* 297:1831, 2002; Fire *et al* (1998) *Nature* 397:806-11, 2002; Moss, *CurrBiol* ii:R772-5, 2002:Bmmmelkamp *et al, supra;* Bass, *Nature 411* 428-9, 2001; Elbashir *et al, Nature 411:494-8;* US Pat. 6,506,559; Published US Pat App. 20030206887; and PCT applications WO99/07409, WO99/32619, WO 00/01846, WO 00/44914, WO00/44895, WO01/29058, WO01/36646, WO01/75164, WO01/92513, WO 01/29058, WO01/89304, WO01/90401, WO02/16620, and WO02/29858.

Ribozymes and siNAs can take any of the forms, including modified versions, described for antisense nucleic acid molecules; and they can be introduced into cells as oligonucleotides (single or double stranded), or in the form of an expression vector.

In a preferred embodiment, an antisense nucleic acid, siNA *(e.g.,* siRNA) or ribozyme comprises a single stranded polynucleotide comprising a sequence that is at least about 90% *(e.g.,* at least about 93%, 95%, 97%, 98% or 99%) identical to a target segment (such as those indicted for Bak and Bax above) or a complement thereof. As used herein, a DNA and an RNA encoded by it are said to contain the same "sequence," taking into account that the thymine bases in DNA are replaced by uracil bases in RNA.

Active variants *(e.g.,* length variants, including fragments; and sequence variants) of the nucleic acid-based inhibitors discussed herein are also within the scope of the invention. An "active" variant is one that retains an activity of the inhibitor from which it is derived (preferably the ability to inhibit expression). It is routine to test a variant to determine for its activity using conventional procedures.

As for length variants, an antisense nucleic acid or siRNA may be of any length that is effective for inhibition of a gene of interest. Typically, an antisense nucleic acid is between about 6 and about 50 nucleotides *(e.g.,* at least about 12, 15, 20, 25, 30, 35, 40, 45 or 50 nt), and may be as long as about 100 to about 200 nucleotides or more. Antisense nucleic acids having about the same length as the gene or coding sequence to be inhibited may be used. When referring to length, the terms bases and base pairs (bp) are used interchangeably, and will be understood to correspond to single stranded (ss) and double stranded (ds) nucleic acids. The length of an effective siNA is generally between about 15 bp and about 29 bp in length, preferably between about 19 and about 29 bp *(e.g.,* about 15, 17, 19, 21, 23, 25, 27 or 29 bp), with shorter and longer sequences being acceptable. Generally, siNAs are shorter than about 30

bases to prevent eliciting interferon effects. For example, an active variant of an siRNA having, for one of its strands, the 19 nucleotide sequence of any of SEQ ID NO: 1, 2, 5 and 6 herein can lack base pairs from either, or both, of ends of the dsRNA; or can comprise additional base pairs at either, or both, ends of the ds RNA, provided that the total of length of the siRNA is between about 19 and about 29 bp, inclusive. One embodiment of the invention is an siRNA that "consists essentially of sequences represented by SEQ ID NO: 1, 2, 5 or 6 or complements of these sequence. The term "consists essentially of is an intermediate transitional phrase, and in this case excludes, for example, sequences that are long enough to induce a significant interferon response. An siRNA of the invention may consist essentially of between about 19 and about 29 bp in length.

As for sequence variants, it is generally preferred that an inhibitory nucleic acid, whether an antisense molecule, a ribozyme (the recognition sequences), or an siNA, comprise a strand that is complementary (100% identical in sequence) to a sequence of a gene that it is designed to inhibit. However, 100% sequence identity is not required to practice the present invention. Thus, the invention has the advantage of being able to tolerate naturally occurring sequence variations, for example, in human c-met, that might be expected due to genetic mutation, polymorphism, or evolutionary divergence. Alternatively, the variant sequences may be artificially generated. Nucleic acid sequences with small insertions, deletions, or single point mutations relative to the target sequence can be effective inhibitors.

The degree of sequence identity may be optimized by sequence comparison and alignment algorithms well-known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters *(e.g.,* University of Wisconsin Genetic Computing Group). At least about 90% sequence identity is preferred *(e.g.,* at least about 92%, 95%, 98% or 99%), or even 100% sequence identity, between the inhibitory nucleic acid and the targeted sequence of targeted gene.

Alternatively, an active variant of an inhibitory nucleic acid of the invention is one that hybridizes to the sequence it is intended to inhibit under conditions of high stringency. For example, the duplex region of an siRNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript under high stringency conditions *(e.g.,* 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50^0C or 70^0C, hybridization for 12-16 hours), followed generally by washing.

DC-I cells or BM-DCs presenting a given antigen X, when not treated with the siRNAs of the invention, respond to sufficient numbers X-specific CD8+ CTL by apoptotic cell death. In contrast, the

same cells transfected with the siRNA or infected with a viral vector encoding the present siRNA sequences survive better despite the delivery of killing signals.

Delivery and expression of the siRNA compositions of the present invention inhibit the death of DCs *in vivo* in the process of a developing T cell response, and thereby promote and stimulate the generation of an immune response induced by immunization with an antigen-encoding DNA vaccine vector. These capabilities have been exemplified by showing that:

(1) co-administration of DNA vaccines encoding HPV-16 E7 with siRNA targeted to Bak and Bax prolongs the lives of antigen-presenting DCs in the draining lymph nodes, thereby enhancing antigen-specific $CD8^+$ T cell responses, and eliciting potent antitumor effects against an E7-expressing tumor in vaccinated subjects.

(2) DCs transfected with siRNA targeting Bak and Bax resist killing by T cells *in vivo*. E7-loaded DCs transfected with Bak/Bax siRNA so that Bak and Bax protein expression is downregulated resist apoptotic death induced by T cells *in vivo*. When administered to subjects, these DCs generate stronger antigen-specific immune responses and manifest therapeutic effects (compared to DCs transfected with control siRNA).

Thus the siRNA constructs of the present invention are useful as a part of nucleic acid vaccination and immunotherapy regimen.

Vectors, Antigen, and IPP Nucleic Acids And Polypeptides

Plasmid Sequences

The sequence of the pcDNA3 plasmid vector (SEQ ID NO: 14) is shown below, followed by the pNGVL4a plasmid vector (SEQ ID NO: 15).

pNGVL4a, a preferred plasmid backbone for the present invention was originally derived from the pNGVL3 vector, which has been approved for human vaccine trials. The pNGVL4a vector includes two immunostimulatory sequences (tandem repeats of CpG dinucleotides) in the noncoding region. Whereas any other plasmid DNA that can transform either APCs, preferably DCs or other cells which, via cross-priming, transfer the antigenic moiety to DCs, is useful in the present invention, pNGFVLA4a is preferred because of the fact that it has already been approved for human therapeutic use.

gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct
gagtagtgcg cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc
cagatatacg cgttgacatt gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata tggagttccg cgttacataa cttacggtaa
atggcccgcc tggctgaccg cccaacgacc cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc attgacgtca atgggtggac
tatttacggt aaactgccca cttggcagta catcaagtgt atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt atgcccagta
catgacctta tgggactttc ctacttggca gtacatctac gtattagtca tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg
actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg
caaatgggcg gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca ctgcttactg gcttatcgaa attaatacga ctcactatag
ggagacccaa gctggctagc gtttaaacgg gccctctaga ctcgagcggc cgccactgtg ctggatatct gcagaattcc accacactgg actagtggat ccgagctcgg
taccaagctt aagtttaaac cgctgatcag cctcgactgt gccttctagt tgccagccat ctgttgtttg cccctccccc gtgccttcct tgaccctgga aggtgccact
cccactgtcc tttcctaata aaatgaggaa attgcatcgc attgtctgag taggtgtcat tctattctgg ggggtggggt ggggcaggac agcaaggggg aggattggga
agacaatagc aggcatgctg gggatgcggt gggctctatg gcttctgagg cggaaagaac cagctggggc tctatgggggt atccccacgc gccctgtagc ggcgcattaa
gcgcggcggg tgtggtggtt acgcgcagcg tgaccgctac acttgccagc gccctagcgc ccgctccttt cgctttcttc ccttcctttc tcgccacgtt cgccggcttt
ccccgtcaag ctctaaatcg gggcatccct ttagggttcc gatttagtgc tttacggcac ctcgacccca aaaaacttga ttagggtgat ggttcacgta gtgggccatc
gccctgatag acggtttttc gccctttgac gttggagtcc acgttcttta atagtggact cttgttccaa actggaacaa cactcaaccc tatctcggtc tattcttttg
atttataagg gattttgggg atttcggcct attggttaaa aaatgagctg atttaacaaa aatttaacgc gaattaattc tgtggaatgt gtgtcagtta gggtgtggaa
agtccccagg ctccccaggc aggcagaagt atgcaaagca tgcatctcaa ttagtcagca accaggtgtg gaaagtcccc aggctcccca gcaggcagaa gtatgcaaag
catgcatctc aattagtcag caaccatagt cccgccccta actccgccca tcccgcccct aactccgccc agttccgccc attctccgcc ccatggctga ctaatttttt
ttatttatgc agaggccgag gccgcctctg cctctgagct attccagaag tagtgaggag gctttttgg aggcctaggc ttttgcaaaa agctcccggg agcttgtata
tccatttttcg gatctgatca agagacagga tgaggatcgt ttcgcatgat tgaacaagat ggattgcacg caggttctcc ggccgcttgg gtggagaggc tattcggcta
tgactgggca caacagacaa tcggctgctc tgatgccgcc gtgttccggc tgtcagcgca ggggcgcccg gttcttttg tcaagaccga cctgtccggt gccctgaatg
aactgcagga cgaggcagcg cggctatcgt ggctggccac gacgggcgtt ccttgcgcag ctgtgctcga cgttgtcact gaagcgggaa gggactggct gctattgggc
gaagtgccgg ggcaggatct cctgtcatct caccttgctc ctgccgagaa agtatccatc atggctgatg caatgcggcg gctgcatacg cttgatccgg ctacctgccc
attcgaccac caagcgaaac atcgcatcga gcgagcacgt actcggatgg aagccggtct tgtcgatcag gatgatctgg acgaagagca tcaggggctc gcgccagccg
aactgttcgc caggctcaag gcgcgcatgc ccgacggcga ggatctcgtc gtgacccatg gcgatgcctg cttgccgaat atcatggtgg aaaatggccg cttttctgga
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caacctgcca tcacgagatt tcgattccac cgccgccttc tatgaaaggt tgggcttcgg aatcgttttc cgggacgccg gctggatgat cctccagcgc ggggatctca
tgctggagtt cttcgcccac cccaacttgt ttattgcagc ttataatggt tacaaataaa gcaatagcat cacaaatttc acaaataaag catttttttc actgcattct
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gcaacgttgt tgccattgct acaggcatcg tggtgtcacg ctcgtcgttt ggtatggctt cattcagctc cggttcccaa cgatcaaggc gagttacatg atcccccatg
ttgtgcaaaa aagcggttag ctccttcggt cctccgatcg ttgtcagaag taagttggcc gcagtgttat cactcatggt tatggcagca ctgcataatt ctcttactgt
catgccatcc gtaagatgct tttctgtgac tggtgagtac tcaaccaagt cattctgaga atagtgtatg cggcgaccga gttgctcttg cccggcgtca atacgggata
ataccgcgcc acatagcaga actttaaaag tgctcatcat tggaaaacgt tcttcggggc gaaaactctc aaggatctta ccgctgttga gatccagttc gatgtaaccc
actcgtgcac ccaactgatc ttcagcatct tttactttca ccagcgtttc tgggtgagca aaaacaggaa ggcaaaatgc cgcaaaaaag ggaataaggg cgacacggaa
atgttgaata ctcatactct tccttttttca atattattga agcatttatc agggttattg tctcatgagc ggatacatat ttgaatgtat ttagaaaaat aaacaaatag
gggttccgcg cacatttccc cgaaaagtgc cacctgacgt c 5431

pNGVA4a vector sequence (SEQ ID NO: 15)

```
tggccattgc atacgttgta tccatatcat aatatgtaca tttatattgg ctcatgtcca acattaccgc catgttgaca ttgattattg actagttatt aatagtaatc
aattacgggg tcattagttc atagcccata tatggagttc cgcgttacat aacttacggt aaatggcccg cctggctgac cgcccaacga cccccgccca ttgacgtcaa
taatgacgta tgttcccata gtaacgccaa tagggacttt ccattgacgt caatgggtgg agtatttacg gtaaactgcc cacttggcag tacatcaagt gtatcatatg
ccaagtacgc cccctattga cgtcaatgac ggtaaatggc ccgcctggca ttatgcccag tacatgacct tatgggactt tcctacttgg cagtacatct acgtattagt
catcgctatt accatggtga tgcggttttg gcagtacatc aatgggcgtg gatagcggtt tgactcacgg ggatttccaa gtctccaccc cattgacgtc aatgggagtt
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ttgtactgag agtgcaccat atgcggtgtg aaataccgca cagatgcgta aggagaaaat accgcatcag attggctat 4479
```

Antigen Polypeptide Sequences

Thus using either CRT or any of the other IPPs, the present invention includes a combined DNA vaccine composition that includes a DNA immunogen encoding E6 with a DNA immunogen encoding E7 along with the delivery of siRNA targeting Bak and/or Bak (or several other pro-apoptotic proteins as described above). The siRNA may be delivered directly *(e.g.,* bound to particles delivered via gene gun) or in the form of a DNA vector that encodes this siRNA.

The E7 nucleic acid sequence (SEQ ID NO: 16) and amino acid sequence (SEQ ID NO: 17) from HPV-16 are shown below (see Accession Number NC_001526)

```
atg cat gga gat aca cct aca ttg cat gaa tat atg tta gat ttg caa cca gag aca act      60
Met His Gly Asp Thr Pro Thr Leu His Glu Tyr Met Leu Asp Leu Gin Pro Glu Thr Thr      20

gat etc tac tgt tat gag caa tta aat gac age tea gag gag gag gat gaa ata gat ggt     120
Asp Leu Tyr cvs Tyr Gi u Gin Leu Asn Asp Ser Ser Glu Glu Glu Asp Glu lie Asp Gly     40

cca get gga caa gca gaa ccg gac aga gee cat tac aat att gta ace ttt tgt tgc aag     180
Pro Ala Gly Gin Ala Glu Pro Asp Arg Ala His Tyr Asn lie val Thr Phe Cys Cys Lys      60

tgt gac tct acg ctt egg ttg tgc gta caa age aca cac gta gac att cgt act ttg gaa     240
cys Asp ser Thr Leu Arg Leu Cys VaI Gin Ser Thr His val Asp lie Arg Thr Leu Glu      80

gac ctg tta atg ggc aca eta gga att gtg tgc ccc ate tgt tct cag gat aag ctt         297
Asp Leu Leu Met Gly Thr Leu Gly lie VaT Cys Pro lie cys ser Gin Asp Lys Leu         99
```

In single letter code, the wild type E7 amino acid sequence is

```
MHGDTPTLHE  YMLDLQPETT  DLYCYEQLND  SSEEEDEIDG  PAGQAEPDRA  HYNIVTFCCK
CDSTLRLCVQ  STHVDIRTLE  DLLMGTLGIV  CPICSQDKL   99  (SEQ ID NO: 17 above))
```

In another embodiment (See GenBank Accession No. AF125673, nucleotides 562-858 and the E7 amino acid sequence) the C-terminal four amino acids QDKL (and their codons) above are replaced with the three amino acids QKP (and the codons cag aaa cca yielding a protein of 98 residues.

When an oncoprotein or an epitope thereof is the immunizing moiety, it is preferable to reduce the tumorigenic risk of the vaccine itself. Because of the potential oncogenicity of the HPV E7 protein, the E7 protein is preferably used in a "detoxified" form

To reduce oncogenic potential of E7 in a construct of this invention, one or more of the following positions of E7 is mutated:

Original residue	Mutant residue	Preferred codon mutation	nt Position (in SEQ ID NO:16)	Amino acid (in SEQ ID NO:17
Cys	Gly (or Ala)	TGT→GGT	70	24
Glu	Gly (or Ala)	GAG→GGG (or GCG)	77	26
Cys	Gly (or Ala)	TGC→GGC	271	91

The preferred E7 (detox) mutant sequence has the following two mutations: a TGT→GGT mutation resulting in a Cys→Gly substitution at position 24 of SEQ ID NO: 17 a and GAG→GGG mutation resulting in a Glu→Gly substitution at position 26 of SEQ ID NO: 17. This mutated amino acid sequence is shown below with the replacement residues underscored.

MHGDTPTLHE YMLDLQPETT DLYGYEGLND SSEEEDEIDG PAGQAEPDRA HYNIVTFCCK
CDSTLRLCVQ STHVDIRTLE DLLMGTLGIV CPICSQKP 97 (SEQIDNO:18)

These substitutions completely eliminate the capacity of the E7 to binding capacity to Rb, and thereby nullify its transforming activity.

Any nucleotide sequence that encodes encoding the above E7 or E7(detox) polypeptide, or an antigenic fragment or epitope thereof, can be used in the present compositions and methods, though the preferred E7 and E7(detox) sequences are shown above.

E6 Protein from HPV-16

The wild type HPV E6 amino acid sequence (see GenBank Accession Number NC_001526) (SEQ ID NO:4) is shown below. This sequence has 158 amino acids.

The wild type E6 nucleotide (SEQ ID NO: 19) and amino acid (SEQ ID NO:20) sequences are shown below (see GenBank accession #'s K02718 and NC_001526)):

atg	cac	caa	aag	aga	act	gca	atg	ttt	cag	gac	cca	cag	gag	cga	ccc	aga	aag	tta	cca	60
Met	His	Gln	Lys	Arg	Thr	Ala	Met	Phe	Gln	Asp	Pro	Gln	Glu	Arg	pro	Arg	Lys	Leu	Pro	*20*
cag	tta	tgc	aca	gag	ctg	caa	aca	act	ata	cat	gat	ata	ata	tta	gaa	tgt	gtg	tac	tgc	120
Gln	Leu	Cys	Thr	Glu	Leu	Gln	Thr	Thr	Ile	His	Asp	Ile	Ile	Leu	Glu	Cys	Val	Tyr	cys	*40*
aag	caa	cag	tta	ctg	cga	cgt	gag	gta	tat	gac	ttt	get	ttt	egg	gat	tta	tgc	ata	gta	180
Lys	Gln	Gln	Leu	Leu	Arg	Arg	Glu	val	Tyr	Asp	Phe	Ala	Phe	Arg	Asp	Leu	Cys	Ile	Val	*60*
tat	aga	gat	ggg	aat	cca	tat	get	gta	tgt	gat	aaa	tgt	tta	aag	ttt	tat	tct	aaa	att	240
Tyr	Arg	Asp	Gly	Asn	Pro	Tyr	Ala	Val	cys	Asp	Lys	cys	Leu	Lys	Phe	Tyr	ser	Lys	Ile	*80*
agt	gag	tat	aga	cat	tat	tgt	tat	agt	ttg	tat	gga	aca	aca	tta	gaa	cag	caa	tac	aac	300
ser	Glu	Tyr	Arg	His	Tyr	cys	Tyr	ser	Leu	Tyr	Gly	Thr	Thr	Leu	Glu	Gln	Gln	Tyr	Asn	*100*
aaa	ccg	ttg	tgt	gat	ttg	tta	att	agg	tgt	att	aac	tgt	caa	aag	cca	ctg	tgt	cct	gaa	360
Lys	Pro	Leu	cys	Asp	Leu	Leu	Ile	Arg	cys	Ile	Asn	cys	Gln	Lys	Pro	Leu	cys	Pro	Glu	*120*
gaa	aag	caa	aga	cat	ctg	gac	aaa	aag	caa	aga	ttc	cat	aat	ata	agg	ggt	egg	tgg	ace	420
Glu	Lys	Gln	Arg	His	Leu	Asp	Lys	Lys	Gln	Arg	Phe	His	Asn	Ile	Arg	Gly	Arg	Trp	Thr	*140*
ggt	cga	tgt	atg	tct	tgt	tgc	aga	tea	tea	aga	aca	cgt	aga	gaa	ace	cag	ctg	*taa*		474
Gly	Arg	cys	Met	Ser	Cys	Cys	Arg	Ser	ser	Arg	Thr	Arg	Arg	Glu	Thr	Gln	Leu	stop		*158*

This polypeptide has 158 amino acids and is shown below in single letter code:

MHQKRTAMFQ DPQERPRKLP QLCTELQTTI HDIILECVYC KQQLLRREVY DFAFRDLCIV
YRDGNPYAVC_ DKCLKFYSKI SEYRHYCYSL YGTTLEQQYN KPLCDLLIRC INCQKPLCPE
EKQRHLDKKQ RFHNIRGRWT GRCMSCCRSS RTRRETQL 158 [SEQ ID NO:20, above]

E6 proteins from cervical cancer-associated HPV types such as HPV-16 induce proteolysis of the p53 tumor suppressor protein through interaction with E6-AP. Human mammary epithelial cells (MECs) immortalized by E6 display low levels of p53. HPV-16 E6 as well as other cancer-related papillomavirus E6 proteins also binds the cellular protein E6BP (ERC-55). As with E7, it is preferred to used a non-oncogenic mutated form of E6, referred to as "E6(detox)." Several different E6 mutations and publications describing them are discussed below.

The preferred amino acid residues to be mutated are underscored in the E6 amino acid sequence above. Some studies of E6 mutants are based upon a shorter E6 protein of 151 nucleic acids, wherein the N-terminal residue was considered to be the Met at position 8 in SEQ ID NO:20 above. That shorter version of E6 is shown below as SEQ ID NO:21.

MFQDPQERPR KLPQLCTELQ TTIHDIILEC VYCKQQLLRR EVYDFAFRDL CIVYRDGNPY
AVCDKCLKFY SKISEYRHYC YSLYGTTLEQ QYNKPLCDLL IRCINCQKPL CPEEKQRHLD
KKQRFHNIRG RWTGRCMSCC RSSRTRRETQ L

To reduce oncogenic potential of E6 in a construct of this invention, one or more of the following positions of E6 is mutated:

Original residue	Mutant residue	aa position in SEQ ID NO:20	aa position in SEQ ID NO:21
Cys	Gly (or Ala)	70	63
Cys	Gly (or Ala)	113	106
Ile	Thr	135	128

Nguyen M *et al, J Virol.* 6:13039-48, 2002, described a mutant of HPV-16 E6 deficient in binding α-helix partners which displays reduced oncogenic potential *in vivo*. This mutant, that involves a replacement of He with Thr as position 128 (of SEQ ID NO:21) , may be used in accordance with the present invention to make an E6 DNA vaccine that has a lower risk of being oncogenic. This Eo($I^{128}T$) mutant is defective in its ability to bind at least a subset of α-helix partners, including E6AP, the ubiquitin ligase that mediates E6-dependent degradation of the p53 protein,

Cassetti MC *et al, Vaccine* 22:520-52, 2004, examined the effects of mutations four or five amino acid positions in E6 and E7 to inactivate their oncogenic potential. The following mutations were examined: Eo-$C^{63}G$ and E6 $C^{106}G$ (positions based on SEQ ID NO:21); E7-$C^{24}G$, E7-$E^{26}G$, and E7 $C^{91}G$ (positions based on SEQ ID NO: 17). Venezuelan equine encephalitis virus replicon particle (VRP) vaccines encoding mutant or wild type E6 and E7 proteins elicited comparable CTL responses and generated comparable antitumor responses in several HPV16 E6(+)E7(+) tumor challenge models: protection from either C3 or TC-I tumor challenge was observed in 100% of vaccinated mice. Eradication of C3 tumors was observed in approximately 90% of the mice. The predicted inactivation of E6 and E7 oncogenic potential was confirmed by demonstrating normal levels of both p53 and Rb proteins in human mammary epithelial cells infected with VRPs expressing mutant E6 and E7 genes.

The HPV 16 E6 protein contains two zinc fingers important for structure and function; one cysteine (C) amino acid position in each pair of C-X-X-C (where X is any amino acid) zinc finger motifs are preferably was mutated at E6 positions 63 and 106 (based on SEQ ID NO:21). Mutants are created, for example, using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). HPV16 E6 containing a single point mutation in the codon for Cys^{106} in SEQ ID NO:21 (= Cys 113 in

SEQ ID NO:20). Cys^{106} neither binds nor facilitates degradation of p53 and is incapable of immortalizing human mammary epithelial cells (MEC), a phenotype dependent upon p53 degradation. A single amino acid substitution at position Cys^{63} of SEQ ID NO:21 (=Cys^{70} in SEQ ID NO:20) destroys several HPV16 E6 functions: p53 degradation, E6TP-1 degradation, activation of telomerase, and, consequently, immortalization of primary epithelial cells.

Any nucleotide sequence that encodes this E6 polypeptide, or preferably, one of the mutants thereof discussed below, or an antigenic fragment or epitope thereof, can be used in the present invention. Other mutations can be tested and used in accordance with the methods described herein including those described in Cassetti *et al, supra.* These mutations can be produced from any appropriate starting sequences by mutation of the coding DNA.

The present invention also includes the use of a tandem E6-E7 vaccine, using one or more of the mutations described herein to render the oncoproteins inactive with respect to their oncogenic potential *in vivo.* VRP vaccines (described in Cassetti *et al, supra)* comprised fused E6 and E7 genes in one open reading frame which were mutated at four or five amino acid positions (see below). Thus, the present constructs may include one or more epitopes of E6 and E7, which may be arranged in their native order or shuffled in any way that permits the expressed protein to bear the E6 and E7 antigenic epitopes in an immunogenic form. DNA encoding amino acid spacers between E6 and E7 or between individual epitopes of these proteins may be introduced into the vector, provided again, that the spacers permit the expression or presentation of the epitopes in an immunogenic manner after they have been expressed by transduced host cells.

Influenza hemagglutinin (HA)

A nucleic acid sequence encoding HA [SEQ ID NO:22] is shown below.

atgaaggcaaacctactggtcctgttaagtgcacttgcagctgcagatgcagacacaatatgtataggctaccatgc
gaacaattcaaccgacactgttgacacagtactcgagaagaatgtgacagtgacacactctgttaacctgctcgaag
acagccacaacggaaaactatgtagattaaaaggaatagccccactacaattggggaaatgtaacatcgccggatgg
ctcttgggaaacccagaatgcgacccactgcttccagtgagatcatggtcctacattgtagaaacaccaaactctga
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aaagattcgaaatatttcccaaagaaagctcatggcccaaccacaacacaaacggagtaacggcagcatgctcccat
gaggggaaaagcagtttttacagaaatttgctatggctgacggagaaggagggctcatacccaaagctgaaaaattc
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atttgaggcaaatggaaatctaatagcaccaatgtatgctttcgcactgagtagaggctttgggtccggcatcatca
cctcaaacgcatcaatgcatgagtgtaacacgaagtgtcaaacaccccctgggagctataaacagcagtctcccttac
cagaatatacacccagtcacaataggagagtgcccaaaatacgtcaggagtgccaaattgaggatggttacaggact
aaggaacactccgtccattcaatccagaggtctatttggagccattgccggttttattgaaggggatggactggaa
tgatagatggatggtatggttatcatcatcagaatgaacagggatcaggctatgcagcggatcaaaaaagcacacaa
aatgccattaacgggattacaaacaaggtgaacactgttatcgagaaaatgaacattcaattcacagctgtgggtaa
agaattcaacaaattagaaaaaaggatggaaaatttaaataaaaaagttgatgatggatttctggacatttggacat
ataatgcagaattgttagttctactggaaaatgaaaggactctggatttccatgactcaaatgtgaagaatctgtat
gagaaagtaaaaagccaattaaagaataatgccaaagaaatcggaaatggatgttttgagttctaccacaagtgtga
caatgaatgcatggaaagtgtaagaaatgggacttatgattatcccaaatattcagaagagtcaaagttgaacaggg
aaaaggtagatggagtgaaattggaatcaatggggatctatcagattctggcgatctactcaactgtcgccagttca
ctggtgcttttggtctccctgggggcaatcagtttctggatgtgttctaatggatctttgcagtgcagaatatgcat
ctga

The amino acid sequence of HA [SEQ ID NO:23; immunodominant epitope underscored, is:

MKANLLVLLS ALMADADTI CIGYHANNST DTVDTVLEKN VTVTHSVNLL EDSHNGKLCR LKGIAPLQLG
KCNIAGWLLG NPECDPLLPV RSWSYIVETP NSENGICYPG DFIDYEELRE QLSSVSSFER FEIFPKESSW
PNHNTNGVTA ACSHEGKSSF YRNLLWLTEK EGSYPKLKNS YVNKKGKEVL VLWGIHHPPN SKEQQNIYQN
ENAYVSVVTS NYNRRFTPEI AERPKVRDQA GRMNYYWTLL KPGDTIIFEA NGNLIAPMYA FALSRGFGSG
HTSNASMHE CNTKCQTPLG AINSSLPYQN IHPVTIGECP KYVRSAKLRM VTGLRNTPSI QSRGLFGAIA
GFIEGGWTGM IDGWYGYHHQ NEQGSGYAAD QKSTQNAING ITNKVNTVIE KMNIQFTAVG KEFNKLEKRM
ENLNKKVDDG FLDIWTYNAE LLVLLENERT LDFHDSNVKN LYEKVKSQLK NNAKEIGNGC FEFYHKCDNE
CMESVRNGTY DYPKYSEESK LNREKVDGVK LESMGIYQIL AIYSTVASSL VLLVSLGAIS FWMCSNGSLQ
CRICI

Other Antigens Associated with Pathogens

A major use for the present invention is as a therapeutic vaccine for cancer and for major chronic viral infections that cause morbidity and mortality worldwide. Such vaccines are designed to eliminate infected cells - this requires T cell responses as antibodies are often ineffective. The vaccines of the present invention are designed to meet these needs.

Preferred antigens are epitopes of pathogenic microorganisms against which the host is defended by effector T cells responses, including CTL and delayed type hypersensitivity. These typically include viruses, intracellular parasites such as malaria, and bacteria that grow intracellular^ such as Mycobacteria and Listeria species. Thus, the types of antigens included in the vaccine compositions of this invention are any of those associated with such pathogens (in addition, of course, to tumor-specific antigens). It is noteworthy that some viral antigens are also tumor antigens in the case where the virus is a causative factor in cancer.

In fact, the two most common cancers worldwide, hepatoma and cervical cancer, are associated with viral infection. Hepatitis B virus(HBV) (Beasley, R.P. *et al., Lancet 2:* 1129-1 133 (1981) has been implicated as etiologic agent of hepatomas. 80-90% of cervical cancers express the E6 and E7 antigens (discussed above and exemplified herein) from one of four "high risk" human papillomavirus types: HPV- 16, HPV- 18, HPV-31 andHPV-45 (Gissmann, L. *etal, Ciba Found Symp. 120:190-207,* 1986; Beaudenon, S., *et al. Nature 321:246-9,* 1986). The HPV E6 and E7 antigens are the most promising targets for virus associated cancers in immunocompetent individuals because of their ubiquitous expression in cervical cancer. In addition to their importance as targets for therapeutic cancer vaccines, virus associated tumor antigens are also ideal candidates for prophylactic vaccines. Indeed, introduction of prophylactic HBV vaccines in Asia have decreased the incidence of hepatoma (Chang, MH *et al. New Engl. J. Med.* 336, 1855-1859 (1997), representing a great impact on cancer prevention.

Among the most important viruses in chronic human viral infections are HPV, HBV, hepatitis C Virus (HCV), human immunodeficiency virus (HfV-I and HTV-2), herpesviruses such as Epstein Barr Virus (EBV),cytomegalovirus (CMV) and HSV-I and HSV-2 and influenza virus. Useful antigens include HBV surface antigen or HBV core antigen; ppUL83 or pp89 of CMV; antigens of gpl20, gp41

or p24 proteins of HTV-I; ICP27, gD2, gB of HSV; or influenza hemagglutinin or nucleoprotein (Anthony, LS *et al, Vaccine* 1999; 17:373-83). Other antigens associated with pathogens that can be utilized as described herein are antigens of various parasites, includes malaria, preferably malaria peptide based on repeats of NANP.

In addition to its applicability to human cancer and infectious diseases, the present invention is also intended for use in treating animal diseases in the veterinary medicine context. Thus, the approaches described herein may be readily applied by one skilled in the art to treatment of veterinary herpesvirus infections including equine herpesviruses, bovine viruses such as bovine viral diarrhea virus (for example, the E2 antigen), bovine herpesviruses, Marek's disease virus in chickens and other fowl; animal retroviral and lentiviral diseases *(e.g.,* feline leukemia, feline immunodeficiency, simian immunodeficiency viruses, *etc.);* pseudorabies and rabies; and the like.

As for tumor antigens, any tumor-associated or tumor-specific antigen that can be recognized by T cells, preferably by CTL, can be used. These include, without limitation, mutant p53, HER2/neu or a peptide thereof, or any of a number of melanoma-associated antigens such as MAGE-I, MAGE-3, MART-1/Melan-A, tyrosinase, gp75, gplOO, BAGE, GAGE-I, GAGE-2, GnT-V, and pl5 (see, for example, US Pat. 6,187,306).

DNA Encoding Immunogenicity-Potentiating Polypeptides (IPPs)

The present inventors and their colleagues have described a number of IPPs and their use in DNA vaccines, in the following publications, all of which are incorporated by reference in their entirety: Kim TW *et at, J Clin Invest 112:* 109-1 17, 2003; Cheng WF *et al, J Clin Invest 108:* 669-678, 2001; Hung CF *et al, Cancer Res* (57:3698-3703, 2001; Chen CH *et al, 2000, supra;* US Pat. 6,734,173; published patent applications WO05/081716, WO05/047501, WO03/085085, WO02/12281, WO02/074920, WO02/061 113, WO02/09645, and WO01/29233. Recently, they have described comparative studies of these IPPs using HPV E6 as the antigen in Peng, S. *et al, J Biomed Sci. 12:689-*700 2005

The DNA sequence encoding the E7 protein fused to the translocation Signal sequence and LAMP-I domain (Sig-E7-Ll) [SEQ ID NO:24] is:

ATGGCGGCCCCCGGCGCCCGGCGGCCGCTGCTCCTGCTGCTGCTGGCAGGCCTTGCACATGGCGCCTCAGCACTCTT
TGAGGATCTAATCATGCATGGAGATACACCTACATTGCATGAATATATGTTAGATTTGCAACCAGAGACAACTGATC
TCTACTGTTATGAGCAATTAAATGACAGCTCAGAGGAGGAGGATGAAATAGATGGTCCAGCTGGACAAGCAGAACCG
GACAGAGCCCATTACMTATTGTTACCTTTTGTTGCAAGTGTGACTCTACGCTTCGGTTGTGCGTACAAAGCACACA
CGTAGACATTCGTACTTTGGAAGACCTGTTAATGGGCACACTAGGAATTGTGTGCCCCATCTGTTCTCAGGATCTTA
ACAACATGTTGATCCCCATTGCTGTGGGCGGTGCCCTGGCAGGGCTGGTCCTCATCGTCCTCATTGCCTACCTCATT
GGCAGGAAGAGGAGTCACGCCGGCTATCAGACCATCTAG

The amino acid sequence of Sig-E7-Ll [SEQ ID NO:25] is:

MAAPGARRPL LLLLLAGLAH GASALFEDLI MHGDTPTLHE YMLDLQPETT DLYCYEQLND
SSEEEDEIDG PAGQAEPDRA HYNIVTFCCK CDSTLRLCVQ STHVDIRTLE DLLMGTLGIV
CPICSQDLNN MLIPIAVGGA LAGLVLIVLI AYLIGRKRSH AGYQTI

The nucleotide sequence of the immunogenic vector pcDNA3-sigE7-Ll [SEQ ID NO:26] is shown below with the SigE7-Ll coding sequence in lower case and underscored:

GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTAT
CTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGA
CMTTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACGCGTTGACATT
GATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAA
CTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGT
AACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGT
ATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTA
TGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAA
TGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTG TTTTTGGCACC
AAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAG
GTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAG
GGAGACCCAAGCTGGCTAGCGTTTAAACGGGCCCTCTAGACTCGAGCGGCCGCCACTGTGCTGGATATCTGCAGAATTCa
tggcggcccccggcgcccggcggccgctgctcctgctgctgctggcaggccttgcacatggcgcctcagcactctttgag
gatctaatcatgcatggagatacacctacattgcatgaatatatgttagatttgcaaccagagacaactgatctctactg
ttatgagcaattaaatgacagctcagaggaggaggatgaaatagatggtccagctggacaagcagaaccggacagagccc
attacaatattgttacctttttgttgcaagtgtgactctacgcttcggttgtgcgtacaaagcacacacgtagacattcgt
actttggaagacctgttaatgggcacactaggaattgtgtgccccatctgttctcaggatcttaacaacatgttgatccc
cattgctgtgggcggtgccctggcagggctggtcctcatcgtcctcattgcctacctcattggcaggaagaggagtcacg
CCggctatcagaccatCtag GGATCCGAGCTCGGTACCAAGCTTAAGTTTAAACCGCTGATCAGCCTCGACTGTGCCTTC
TAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCT
AATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGCAGGACAGCAAG
GGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTG
GGGCTCTAGGGGGTATCCCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCG
CTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGT
CAAGCTCTAAATCGGGGCATCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGG
TGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGG TTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTG
GACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGGGGATTTCG
GCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTAATTCTGTGGAATGTGTGTCAGTTAGGGTGT
GGAAAGTCCCCAGGCTCCCCAGGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGT
CCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCG
CCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAAT TTTTTTTTATTTATGCAGAGGC
CGAGGCCGCCTCTGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGC TTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTCC
CGGGAGCTTGTATATCCATTTTCGGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTG
CACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGC
CGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTC TTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGC
AGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCG
GGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATC
CATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCA
TCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCA
GCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCC
GAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACA
TAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCC
GCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACC
GACCAAGCGACGCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGT
TTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCCAACTTGTTTATTG
CAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCA TTTTTTTTCACTGCATTCTAGTTGT
GGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGTATACCGTCGACCTCTAGAGCTTGGCGTAATCATGGTC
ATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCT
GGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGC
CAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGA
CTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAG
GGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTT
TTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATA
AAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCG
CCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCC
AAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCC
GGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAG
AGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACC
TTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGG TTTTTTTGTTTGCAAGCAGCA
GATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACT
CACGTTMGGGATTTTGGTCATGAGATTATCAAAMGGATCTTCACCTAGATCCTTTTAAATTAAAAATGMGTTTTAAA
TCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTAC:CAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTG

TCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCA
GTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAG
CGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCC
AGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCA
GCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCG
ATCGTTGTCAGAA GTAA GTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCC
ATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCT
CTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCG
GGG CGAA AACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGC
ATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACAC
GGAAATGTTGAATACTCATACTCTTCC TTTTTrCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATAC
ATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC

HSP70 from *M .tuberculosis*

The nucleotide sequence encoding HSP70 (SEQ ID NO: 27) is shown below (nucleotides 10633-12510 of the *M .tuberculosis* genome in GenBank NC 000962);

atggctcg tgcggtcggg atcgacctcg ggaccaccaa ctccgtcgtc tcggttctgg aaggtggcga
cccggtcgtc gtcgccaact ccgagggctc caggaccacc ccgtcaattg tcgcgttcgc ccgcaacggt
gaggtgctgg tcggccagcc cgccaagaac caggcagtga ccaacgtcga tcgcaccgtg cgctcggtca
agcgacacat gggcagcgac tggtccatag agattgacgg caagaaatac accgcgccgg agatcagcgc
ccgcattctg atgaagctga agcgcgacgc cgaggcctac ctcggtgagg acattaccga cgcggttatc
acgacgcccg cctacttcaa tgacgcccag cgtcaggcca ccaaggacgc cggccagatc gccggcctca
acgtgctgcg gatcgtcaac gagccgaccg cggccgcgct ggcctacggc ctcgacaagg gcgagaagga
gcagcgaatc ctggtcttcg acttgggtgg tggcactttc gacgtttccc tgctggagat cggcgagggt
gtggttgagg tccgtgccac ttcgggtgac aaccacctcg gcggcgacga ctgggaccag cgggtcgtcg
attggctggt ggacaagttc aagggcacca gcggcatcga tctgaccaag gacaagatgg cgatgcagcg
gctgcgggaa gccgccgaga aggcaaagat cgagctgagt tcgagtcagt ccacctcgat caacctgccc
tacatcaccg tcgacgccga caagaacccg ttgttcttag acgagcagct gacccgcgcg gagttccaac
ggatcactca ggacctgctg gaccgcactc gcaagccgtt ccagtcggtg atcgctgaca ccggcatttc
ggtgtcggag atcgatcacg ttgtgctcgt gggtggttcg acccggatgc ccgcggtgac cgatctggtc
aaggaactca ccggcggcaa ggaacccaac aagggcgtca accccgatga ggttgtcgcg gtgggagccg
ctctgcaggc cggcgtcctc aagggcgagg tgaaagacgt tctgctgctt gatgttaccc cgctgagcct
gggtatcgag accaagggcg gggtgatgac caggctcatc gagcgcaaca ccacgatccc caccaagcgg
tcggagactt tcaccaccgc cgacgacaac caaccgtcgg tgcagatcca ggtctatcag ggggagcgtg
agatcgccgc gcacaacaag ttgctcgggt ccttcgagct gaccggcatc ccgccggcgc cgcgggggat
tccgcagatc gaggtcactt tcgacatcga cgccaacggc attgtgcacg tcaccgccaa ggacaagggc
accggcaagg agaacacgat ccgaatccag gaaggctcgg gcctgtccaa ggaagacatt gaccgcatga
tcaaggacgc cgaagcgcac gccgaggagg atcgcaagcg tcgcgaggag gccgatgttc gtaatcaagc
cgagacattg gtctaccaga cggagaagtt cgtcaaagaa cagcgtgagg ccgagggtgg ttcgaaggta
cctgaagaca cgctgaacaa ggttgatgcc gcggtggcgg aagcgaaggc ggcacttggc ggatcggata
tttcggccat caagtcggcg atggagaagc tgggccagga gtcgcaggct ctggggcaag cgatctacga
agcagctcag gctgcgtcac aggccactgg cgctgcccac cccggcggcg agccgggcgg tgcccacccc
ggctcggctg atgacgttgt ggacgcggag gtggtcgacg acggccggga ggccaagtga

The amino acid sequence of HSP70 [SEQ ID NO:28] is:

MARAVGIDLG TTNSVVSVLE GGDPVVVANS EGSRTTPSIV AFARNGEVLV GQPAKNQAVT NVDRTVRSVK
RHMGSDWSIE IDGKKYTAPE ISARILMKLK RDAEAYLGED ITDAVITTPA YFNDAQRQAT KDAGQIAGLN
VLRIVNEPTA AALAYGLDKG EKEQRILVFD LGGGTFDVSL LEIGEGVVEV RATSGDNHLG GDDWDQRVVD
WLVDKFKGTS GIDLTKDKMA MQRLREAAEK AKIELSSSQS TSINLPYITV DADKNPLFLD EQLTRAEFQR
ITQDLLDRTR KPFQSVIADT GISVSEIDHV VLVGGSTRMP AVTDLVKELT GGKEPNKGVN PDEVVAVGAA
LQAGVLKGEV KDVLLLDVTP LSLGIETKGG VMTRLIERNT TIPTKRSETF TTADDNQPSV QIQVYQGERE
IAAHNKLLGS FELTGIPPAP RGIPQIEVTF DIDANGIVHV TAKDKGTGKE NTIRIQEGSG LSKEDIDRMI
KDAEAHAEED RKRREEADVR NQAETLVYQT EKFVKEQREA EGGSKVPEDT LNKVDAAVAE AKAALGGSDI
SAIKSAMEKL GQESQALGQA IYEAAQAASQ ATGAAHPGGE PGGAHPGSAD DVVDAEVVDD GREAK

The E7-Hsp70 Chimera/Fusion Polypeptide (Nucleotide sequence SEQ ID NO:29 and amino acid sequence SEQ ID NO:30) are provided below. The E7 coding sequence is shown in upper case and underscored.

1/1 31/11
ATG CAT GGA GAT ACA CCT ACA TTG CAT GAA TAT ATG TTA GAT TTG CAA CCA GAG ACA ACT
Met His Gly Asp Thr Pro Thr Leu His GlU Tyr Met Leu ASP Leu Gin Pro GlU Thr Thr
61/21 91/31
GAT CTC TAC TGT TAT GAG CAA TTA AAT GAC AGC TCA GAG GAG GAG GAT GAA ATA GAT GGT
Asp Leu Tyr Cys Tyr Glu Gin Leu Asn Asp Ser Ser GlU Glu GlU Asp Glu H e ASP Gly
121/41 151/51
CCA GCT GGA CAA GCA GAA CCG GAC AGA GCC CAT TAC AAT ATT GTA ACC TTT TGT TGC AAG
pro Ala Gly Gin Ala GlU Pro Asp Arg Ala His Tyr Asn H e val Thr Phe Cys cys Lys
181/61 211/71
TGT GAC TCT ACG CTT CGG TTG TGC GTA CAA AGC ACA CAC GTA GAC ATT CGT ACT TTG GAA
cys Asp ser Thr Leu Arg Leu cys val Gin Ser Thr HIS val Asp H e Arg Thr Leu GlU
241/81 271/91
GAC CTG TTA ATG GGC ACA CTA GGA ATT GTG TGC CCC ATC TGT TCT CAA GGA TCC atg get
Asp Leu Leu Met Gly Thr Leu Gly H e val cys Pro H e cys Ser Gin Gly ser Met Ala
301/101 331/111
cgt gcg gtc ggg ate gac etc ggg ace ace aac tec gtc gtc teg gtt ctg gaa ggt ggc
Arg Ala val GIy lie Asp Leu GIy Thr Thr Asn Ser val val Ser Val Leu Glu GTy GTy
361/121 391/131
gac ccg gtc gtc gtc gcc aac tec gag ggc tec agg ace ace ccg tea att gtc gcg ttc
Asp Pro Val val Val Ala Asn Ser GlU Gly ser Arg Thr Thr pro ser H e Val Ala Phe
421/141 451/151
gcc cgc aac ggt gag gtg ctg gtc ggc cag ccc gcc aag aac cag gca gtg ace aac gtc
Ala Arg Asn Gly Glu Val Leu val Gly Gin Pro Ala Lys Asn Gin Ala val Thr Asn val
481/161 511/171
gat cgc ace gtg cgc teg gtc aag cga cac atg ggc age gac tgg tec ata gag att gac
Asp Arg Thr val Arg ser Val Lys Arg His Met Gly ser Asp Trp Ser H e Glu H e Asp
541/181 571/191
ggc aag aaa tac ace gcg ccg gag ate age gcc cgc att ctg atg aag ctg aag cgc gac
Gly Lys Lys Tyr Thr Ala Pro Glu lie ser Ala Arg H e Leu Met Lys Leu Lys Arg Asp
601/201 631/211
gcc gag gcc tac etc ggt gag gac att ace gac gcg gtt ate acg acg CCC gcc tac ttc
Ala Glu Ala Tyr Leu Gly GlU Asp H e Thr Asp Ala Val lie Thr Thr Pro Ala Tyr phe
661/221 691/231
aat gac gcc cag cgt cag gcc ace aag gac gcc ggc cag ate gcc ggc etc aac gtg ctg
Asn Asp Ala Gin Arg Gin Ala Thr Lys Asp Ala Gly Gin lie Ala Gly Leu Asn VaT Leu
721/241 751/251
egg ate gtc aac gag ccg ace gcg gcc gcg ctg gcc tac ggc etc gac aag ggc gag aag
Arg lie val Asn Glu Pro Thr Ala Ala Ala Leu Ala Tyr Gly Leu Asp Lys Gly Glu Lys
781/261 811/271
gag cag cga ate ctg gtc ttc gac ttg ggt ggt ggc act ttc gac gtt tec ctg ctg gag
Glu Gin Arg lie Leu val Phe Asp Leu Gly Gly Gly Thr Phe Asp val Ser Leu Leu GlU
841/281 871/291
ate ggc gag ggt gtg gtt gag gtc cgt gcc act teg ggt gac aac cac etc ggc ggc gac
lie Gly Glu Gly val Val GlU Val Arg Ala Thr Ser Gly Asp Asn His Leu Gly GTy Asp
901/301 931/311
gac tgg gac cag egg gtc gtc gat tgg ctg gtg gac aag ttc aag ggc ace age ggc ate
Asp Trp Asp Gin Arg val val Asp Trp Leu val Asp Lys Phe Lys Gly Thr Ser GTy H e
961/321 991/331
gat ctg ace aag gac aag atg gcg atg cag egg ctg egg gaa gcc gcc gag aag gca aag
Asp Leu Thr Lys Asp Lys Met Ala Met Gin Arg Leu Arg Glu Ala Ala Glu Lys Ala Lys
1021/341 1051/351
ate gag ctg agt teg agt cag tec ace teg ate aac ctg CCC tac ate ace gtc gac gcc
lie Glu Leu Ser ser ser Gin Ser Thr Ser lie Asn Leu Pro Tyr H e Thr Val Asp Ala
1081/361 1111/371
gac aag aac ccg ttg ttc tta gac gag cag ctg ace cgc gcg gag ttc caa egg ate act
Asp Lys Asn Pro Leu Phe Leu Asp GlU Gin Leu Thr Arg Ala Glu Phe Gin Arg H e Thr
1141/381 1171/391
cag gac ctg ctg gac cgc act cgc aag ccg ttc cag teg gtg ate get gac ace ggc att
Gin Asp Leu Leu Asp Arg Thr Arg Lys Pro Phe Gin ser val lie Ala Asp Thr GTy H e
1201/401 1231/411
teg gtg teg gag ate gat cac gtt gtg etc gtg ggt ggt teg ace egg atg CCC gcg **gtg**
ser val ser Glu H e Asp His Val VaT Leu val Gly Gly Ser Thr Arg Met Pro Ala **Val**
1261/421 1291/431
ace gat ctg gtc aag gaa etc ace ggc ggc aag gaa CCC aac aag ggc gtc aac CCC gat
Thr Asp Leu val Lys Glu Leu Thr Gly Gly Lys Glu Pro Asn Lys Gly val Asn Pro Asp
1321/441 1351/451
gag gtt gtc gcg gtg gga gcc get ctg cag gcc ggc gtc etc aag ggc gag gtg aaa gac
Glu val val Ala val Gly Ala Ala Leu Gin Ala Gly Val Leu Lys Gly Glu VaT Lys Asp
1381/461 1411/471
gtt ctg ctg ctt gat gtt ace ccg ctg age ctg ggt ate gag ace aag ggc ggg gtg atg
val Leu Leu Leu Asp Val Thr Pro Leu Ser Leu Gly H e GlU Thr Lys Gly Gly VaT Met
1441/481 1471/491

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acc agg etc ate gag cgc aac acc acg ate ccc acc aag egg teg gag act ttc acc acc
Thr Arg Leu lie Glu Arg Asn τhr Thr lie Pro Thr Lys Arg Ser Glu Thr Phe Thr Thr
1501/501                                    1531/511
gcc gac gac aac caa ccg teg gtg cag ate cag gtc tat cag ggg gag cgt gag ate gcc
Ala Asp Asp Asn Gin Pro ser VaI Gin lie Gin VaI Tyr Gin Gly Glu Arg Glu lie Ala
1561/521                                    1591/531
gcg cac aac aag ttg etc ggg tec ttc gag ctg acc ggc ate ccg ccg gcg ccg egg ggg
Ala His Asn Lys Leu Leu GIy Ser Phe Glu Leu Thr Gly lie Pro Pro Ala Pro Arg Gly
1621/541                                    1651/551
att ccg cag ate gag gtc act ttc gac ate gac gcc aac ggc att gtg cac gtc acc gcc
lie Pro Gin lie Glu VaI Thr Phe Asp lie Asp Ala Asn Gly lie VaI His val Thr Ala
1681/561                                    1711/571
aag gac aag ggc acc ggc aag gag aac acg ate cga ate cag gaa ggc teg ggc ctg tec
Lys Asp Lys Gly Thr Gly Lys Glu Asn Thr lie Arg lie Gin Glu Gly ser Gly Leu ser
1741/581                                    1771/591
aag gaa gac att gac cgc atg ate aag gac gcc gaa gcg cac gcc gag gag gat cgc aag
Lys Glu Asp lie Asp Arg Met lie Lys Asp Ala Glu Ala His Ala Glu Glu Asp Arg Lys
1801/601                                    1831/611
cgt cgc gag gag gcc gat gtt cgt aat caa gcc gag aca ttg gtc tac cag acg gag aag
Arg Arg Glu Glu Ala Asp VaI Arg Asn Gin Ala Glu Thr Leu VaI Tyr Gin Thr Glu Lys
1861/621                                    1891/631
ttc gtc aaa gaa cag cgt gag gcc gag ggt ggt teg aag gta cct gaa gac acg ctg aac
Phe VaI Lys Glu Gin Arg Glu Ala Glu Gly G ly Ser Lys val Pro Glu Asp Thr Leu Asn
1921/641                                    1951/651
aag gtt gat gcc gcg gtg gcg gaa gcg aag gcg gca ctt ggc gga teg gat att teg gcc
Lys VaI Asp Ala Ala VaT Ala Glu Ala Lys Ala Ala Leu GIy Gly Ser Asp lie ser Ala
1981/661                                    2011/671
ate aag teg gcg atg gag aag ctg ggc cag gag teg cag get ctg ggg caa gcg ate tac
lie Lys Ser Ala Met Glu Lys Leu Gly Gin Glu ser Gin Ala Leu GIy Gin Ala lie Tyr
2041/681                                    2071/691
gaa gca get cag get gcg tea cag gcc act ggc get gcc cac ccc ggc teg get gat gaA
GLU ALA ALA GLN ALA ALA SER GLN ALA THR GLY ALA ALA HIS PRO GLY SER ALA ASP GLU
2101/701
AGC a
ser
```

ETA(dff) from *Pseudomonas aeruginosa*

The complete coding sequence for *Pseudomonas aeruginosa* exotoxin type A (ETA) - SEQ ID NO:31-GenBank Accession No. K01397, is shown below:

```
ctgcagctgg tcaggccgtt tccgcaacgc ttgaagtcct ggccgatata ccggcagggc cagccatcgt
tcgacgaata aagccacctc agccatgatg ccctttccat ccccagcgga accccgacat ggacgccaaa
gccctgctcc tcggcagcct ctgcctggcc gccccattcg ccgacgcggc gacgctcgac aatgctctct
ccgcctgcct cgccgcccgg ctcggtgcac cgcacacggc ggagggccag ttgcacctgc cactcaccct
tgaggcccgg cgctccaccg gcgaatgcgg ctgtacctcg gcgctggtgc gatatcggct gctggccagg
ggcgccagcg ccgacagcct cgtgcttcaa gagggctgct cgatagtcgc caggacacgc cgcgcacgct
gaccctggcg gcggacgccg gcttggcgag cggccgcgaa ctggtcgtca ccctgggttg tcaggcgcct
gactgacagg ccgggctgcc accaccaggc cgagatggac gccctgcatg tatcctccga tcggcaagcc
tcccgttcgc acattcacca ctctgcaatc cagttcataa atcccataaa agccctcttc cgctccccgc
cagcctcccc gcatcccgca ccctagacgc cccgccgctc tccgccggct cgcccgacaa gaaaaaccaa
ccgctcgatc agcctcatcc ttcacccatc acaggagcca tcgcgatgca cctgataccc cattggatcc
ccctggtcgc cagcctcggc ctgctcgccg gcggctcgtc cgcgtccgcc gccgaggaag ccttcgacct
ctggaacgaa tgcgccaaag cctgcgtgct cgacctcaag gacggcgtgc gttccagccg catgagcgtc
gacccggcca tcgccgacac caacggccag ggcgtgctgc actactccat ggtcctggag ggcggcaacg
acgcgctcaa gctggccatc gacaacgccc tcagcatcac cagcgacggc ctgaccatcc gcctcgaagg
cggcgtcgag ccgaacaagc cggtgcgcta cagctacacg cgccaggcgc gcggcagttg gtcgctgaac
tggctggtac cgatcggcca cgagaagccc tcgaacatca aggtgttcat ccacgaactg aacgccggca
accagctcag ccacatgtcg ccgatctaca ccatcgagat gggcgacgag ttgctggcga agctggcgcg
cgatgccacc ttcttcgtca gggcgcacga gagcaacgag atgcagccga cgctcgccat cagccatgcc
ggggtcagcg tggtcatggc ccagacccag ccgcgccggg aaaagcgctg gagcgaatgg gccagcggca
aggtgttgtg cctgctcgac ccgctggacg gggtctacaa ctacctcgcc cagcaacgct gcaacctcga
cgatacctgg gaaggcaaga tctaccgggt gctcgccggc aacccggcga agcatgacct ggacatcaaa
cccacggtca tcagtcatcg cctgcacttt cccgagggcg gcagcctggc cgcgctgacc gcgcaccagg
cttgccacct gccgctggag actttcaccc gtcatcgcca gccgcgcggc tgggaacaac tggagcagtg
cggctatccg gtgcagcggc tggtcgccct ctacctggcg gcgcggctgt cgtggaacca ggtcgaccag
gtgatccgca acgccctggc cagccccggc agcggcggcg acctgggcga agcgatccgc gagcagccgg
agcaggcccg tctggccctg accctggccg ccgccgagag cgagcgcttc gtccggcagg gcaccggcaa
cgacgaggcc ggcgcggcca acgccgacgt ggtgagcctg acctgcccgg tcgccgccgg tgaatgcgcg
ggcccggcgg acagcggcga cgccctgctg gagcgcaact atcccactgg cgcggagttc ctcggcgacg
gcggcgacgt cagcttcagc acccgcggca cgcagaactg gacggtggag cggctgctcc aggcgcaccg
```

ccaactggag gagcgcggct atgtgttcgt cggctaccac ggcaccttcc tcgaagcggc gcaaagcatc
gtcttcggcg gggtgcgcgc gcgcagccag gacctcgacg cgatctggcg cggtttctat atcgccggcg
atccggcgct ggcctacggc tacgcccagg accaggaacc cgacgcacgc ggccggatcc gcaacggtgc
cctgctgcgg gtctatgtgc cgcgctcgag cctgccgggc ttctaccgca ccagcctgac cctggccgcg
ccggaggcgg cgggcgaggt cgaacggctg atcggccatc cgctgccgct gcgcctggac gccatcaccg
gccccgagga ggaaggcggg cgcctggaga ccattctcgg ctggccgctg gccgagcgca ccgtggtgat
tccctcggcg atccccaccg acccgcgcaa cgtcggcggc gacctcgacc cgtccagcat ccccgacaag
gaacaggcga tcagcgccct gccggactac gccagccagc ccggcaaacc gccgcgcgag gacctgaagt
aactgccgcg accggccggc tcccttcgca ggagccggcc ttctcggggc ctggccatac atcaggtttt
cctgatgcca gcccaatcga atatgaattc 2760

The amino acid sequence of ETA (SEQ ID NO:32), GenBank Accession No. K01397, is:

MHLIPHWIPL *VASLGLLAGG* *SSASAAEEAF* DLWNECAKAC VLDLKDGVRS SRMSVDPAIA DTNGQGVLHY
SMVLEGGNDA LKLAIDNALS ITSDGLTIRL EGGVEPNKPV RYSYTRQARG SWSLNWLVPI GHEKPSNIKV
FIHELNAGNQ LSHMSPIYTI EMGDELLAKL ARDATFFVRA HESNEMQPTL AISHAGVSVV MAQTQPRREK
RWSEWASGKV LCLLDPLDGV YNYLAQQRCN LDDTWEGKIY RVLAGNPAKH DLDIKPTVIS HRLHFPEGGS
LAALTAHQAC HLPLETFTRH RQPRGWEQLE QCGYPVQRLV ALYLAARLSW NQVDQVIRNA LASPGSGGDL
GEAIREQPEQ ARLALTLAAA ESERFVRQGT GNDEAGAANA DVVSLTCPVA AGECAGPADS GDALLERNYP
TGAEFLGDGG DVSFSTRGTQ NWTVERLLQA HRQLEERGYV FVGYHGTFLE AAQSIVFGGV RARSQDLDAI
WRGFYIAGDP ALAYGYAQDQ EPDARGRIRN GALLRVYVPR SSLPGFYRTS LTLAAPEAAG EVERLIGHPL
PLRLDAITGP EEEGGRLETI LGWPLAERTV VIPSAIPTDP RNVGGDLDPS SIPDKEQAIS ALPDYASQPG
KPPREDLK 638

Residues 1-25 (italicized) above represent the signal peptide. The first residue of the mature polypeptide, Ala, is bolded/underscored. The mature polypeptide is residues 26-638 of SEQ ID NO:32.

Domain π (ETA(II)), translocation domain (underscored above) spans residues 247-417 of the mature polypeptide (corresponding to residues 272-442 of SEQ ID NO:32) and is presented below separately as SEQ ID NO:33.

RLHFPEGGSL AALTAHQACH LPLETFTRHR QPRGWEQLEQ CGYPVQRLVA LYLAARLSWN QVDQVIRNAL
ASPGSGGDLG EAIREQPEQA RLALTLAAAE SERFVRQGTG NDEAGAANAD VVSLTCPVAA GECAGPADSG
DALLERNYPT GAEFLGDGGD VSFSTRGTQN W 171

The construct in which ETA(dII) is fused to HPV-16 E7 is shown below (nucleotides; SEQ ID NO:34 and amino acids; SEQ ID NO:35). The ETA(dII) sequence appears in plain font, extra codons from plasmid pcDNA3 are italicized. Nucleotides between ETA(dII) and E7 are also bolded (and result in the interposition of two amino acids between ETA(dII) and E7). The E7 amino acid sequence is underscored (ends with Gln at position 269).

```
1/1                                                                 31/11
atg cgc ctg cac ttt ccc gag ggc ggc age ctg gcc gcg ctg ace gcg cac cag get tgc
Met arg leu his phe pro glu gly gly ser leu a!a ala leu thr ala his gin ala cys
61/21                                                               91/31
cac ctg ccg ctg gag act ttc ace cgt cat cgc cag ccg cgc ggc tgg gaa caa ctg gag
His Leu Pro Leu Glu Thr Phe Thr Arg His Arg Gin Pro Arg Gly Trp Glu Gin Leu Glu
121/41                                                              151/51
cag tgc ggc tat ccg gtg cag egg ctg gtc gcc etc tac ctg gcg gcg egg ctg teg tgg
Gin cys Gly Tyr Pro VaT Gin Arg Leu val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp
181/61                                                              211/71
aac cag gtc gac cag gtg ate cgc aac gcc ctg gcc age ccc ggc age ggc ggc gac ctg
Asn Gin VaI Asp Gin val lie Arg Asn Ala Leu Ala ser Pro Gly Ser Gly Gly Asp Leu
241/81                                                              271/91
ggc gaa gcg ate cgc gag cag ccg gag cag gcc cgt ctg gcc ctg ace ctg gcc gcc gcc
Gly Glu Ala lie Arg Glu Gin Pro Glu Gin Ala Arg Leu Ala Leu Thr Leu Ala Ala Ala
301/101                                                             331/111
gag age gag cgc ttc gtc egg cag ggc ace ggc aac gac gag gcc ggc gcg gcc aac gcc
Glu Ser Glu Arg Phe val Arg Gin Gly Thr Gly Asn Asp Glu Ala GTy Ala Ala Asn Ala
361/121                                                             391/131
gac gtg gtg age ctg ace tgc ccg gtc gcc gcc ggt gaa tgc gcg ggc ccg gcg gac age
Asp VaT VaI ser Leu Thr Cys Pro Val Ala Ala Gly Glu cys Ala Gly Pro Ala Asp ser
421/141                                                             451/151
ggc gac gcc ctg ctg gag cgc aac tat ccc act ggc gcg gag ttc etc ggc gac ggc ggc
Gly Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr Gly Ala Glu Phe Leu GTy Asp GTy GTy
481/161                                                             511/171
gac gtc age ttc age ace cgc ggc acg cag aac gaa ttc atg cat gga gat aca cct aca
Asp VaI Ser Phe ser Thr Arg Gly Thr Gin Asn Glu Phe Met His GTV ASP Thr Pro Thr
541/181                                                             571/191
ttg cat gaa tat atg tta gat ttg caa cca gag aca act gat etc tac tgt tat gag caa
Leu His Glu Tyr Met Leu ASP Leu Gin Pro Glu Thr Thr Asp Leu Tyr cvs Tyr Glu Gin
601/201                                                             631/211
tta aat gac age tea gag gag gag gat gaa ata gat ggt cca get gga caa gca gaa ccg
Leu Asn ASP ser ser Glu Glu Glu Asp Glu lie Asp GTV Pro Ala GTV Gin Ala Glu Pro
661/221                                                             691/231
gac aga gcc cat tac aat att gta ace ttt tgt tgc aag tgt gac tct acg ctt egg ttg
Asp Arg Ala His Tyr Asn H e val Thr Phe Cvs Cvs Lvs cvs Asp Ser Thr Leu Arg Leu
721/241                                                             751/251
tgc gta caa age aca cac gta gac att cgt act ttg gaa gac ctg tta atg ggc aca eta
Cvs val Gin Ser Thr His val ASP lie Arg Thr Leu Glu Asp Leu Leu Met GTV Thr Leu
781/261                                                             811/271
gga att gtg tgc ccc ate tgt tct caa gga tec gag etc ggt ace aag ctt aag ttt aaa
GTV lie VaT cvs Pro lie cvs Ser Gin Gly ser Glu Leu Gly Thr LyS Leu Lys Phe Lys
841/281
ccg ctg ate age etc gac tgt gcc ttc tag
Pro Leu lie ser Leu Asp Cys Ala Phe AMB
```

The nucleotide sequence of the pcDNA3 vector encoding E7 and HSP70 (**pcDNA3-E7-Hsp70**) (SEQ ID NO:36) is shown below.: The E7-Hsp70 fusion sequence is shown in upper case, underscored. Plasmid sequences are in lower case.

```
[  10     I   20    ]  30    1   40    I   50    1   60    I   70    I   80    1   90    1
gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg ccgcatagtt aagccagtat ctgctccctg
cttgtgtgtfc ggaggtcgct gagtagtgcg cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt gattattgac tagttattaa tagtaatcaa
ttacggggtc attagttcat agcccatata tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc
cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc attgacgtca atgggtggac tatttacggt
aaactgccca ottggcagta catcaagtgt atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca tcgctattao catggtgatg cggttttggc
agtacatcaa tgggcgtgga tagcggtttg actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc
aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg gtaggcgtgt acggtgggag gtctatataa
gcagagctct ctggctaact agagaaccca ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gctggctagc
gtttaaacgg gccctctaga ctcgagcggc cgccactgtg ctggatatct gcagaattcc accacactgg actagtggat ccATGCATGG
AGATACACCT ACATTGCATG AATATATGTT AGATTTGCAA CCAGAGACAA CTGATCTCTA CTGTTATGAG CAATTAAATG ACAGCTCAGA
GGAGGAGGAT GAAATAGATG GTCCAGCTGG ACAAGCAGAA CCGGACAGAG CCCATTACAA TATTGTAACC TTTTGTTGCA AGTGTGACTC
TACGCTTCGG TTGTGCGTAC AAAGCACACA CGTAGACATT CGTACTTTGG AAGACCTGTT AATGGGCACA CTAGGAATTG TGTGCCCCAT
CTGTTCTCAA GGATCCATGG CTCGTGCGGT CGGGATCGAC CTCGGGACCA CCAACTCCGT CGTCTCGGTT CTGGAAGGTG GCGACCCGGT
CGTCGTCGCC AACTCCGAGG GCTCCAGGAC CACCCCGTCA ATTGTCGCGT TCGCCCGCAA CGGTGAGGTG CTGGTCGGCC AGCCCGCCAA
GAACCAGGCA GTGACCAACG TCGATCGCAC CGTGCGCTCG GTCAAGCGAC ACATGGGCAG CGACTGGTCC ATAGAGATTG ACGGCAAGAA
ATACACCGCG CCGGAGATCA GCGCCCGCAT TCTGATGAAG CTGAAGCGCG ACGCCGAGGC CTACCTCGGT GAGGACATTA CCGACGCGGT
TATCACGACG CCCGCCTACT TCAATGACGC CCAGCGTCAG GCCACCAAGG ACGCCGGCCA GATCGCCGGC CTCAACGTGC TGCGGATCGT
CAACGAGCCG ACCGCGGCCG CGCTGGCCTA CGGCCTCGAC AAGGGCGAGA AGGAGCAGCG AATCCTGGTC TTCGACTTGG GTGGTGGCAC
TTTCGACGTT TCCCTGCTGG AGATCGGCGA GGGTGTGGTT GAGGTCCGTG CCACTTCGGG TGACAACCAC CTCGGCGGCG ACGACTGGGA
CCAGCGGGTC GTCGATTGGC TGGTGGACAA GTTCAAGGGC ACCAGCGGCA TCGATCTGAC CAAGGACAAG ATGGCGATGC AGCGGCTGCG
GGAAGCCGCC GAGAAGGCAA AGATCGAGCT GAGTTCGAGT CAGTCCACCT CGATCAACCT GCCCTACATC ACCGTCGACG CCGACAAGAA
CCCGTTGTTC TTAGACGAGC AGCTGACCCG CGCGGAGTTC CAACGGATCA CTCAGGACCT GCTGGACCGC ACTCGCAAGC CGTTCCAGTC
GGTGATCGCT GACACCGGCA TTTCGGTGTC GGAGATCGAT CACGTTGTGC TCGTGGGTGG TTCGACCCGG ATGCCCGCGG TGACCGATCT
GGTCAAGGAA CTCACCGGCG GCAAGGAACC CAACAAGGGC GTCAACCCCG ATGAGGTTGT CGCGGTGGGA GCCGCTCTGC AGGCCGGCGT
CCTCAAGGGC GAGGTGAAAG ACGTTCTGCT GCTTGATGTT ACCCCGCTGA GCCTGGGTAT CGAGACCAAG GGCGGGGTGA TGACCAGGCT
CATCGAGCGC AACACCACGA TCCCCACCAA GCGGTCGGAG ACTTTCACCA CCGCCGACGA CAACCAACCG TCGGTGCAGA TCCAGGTCTA
TCAGGGGGAG CGTGAGATCG CCGCGCACAA CAAGTTGCTC GGGTCCTTCG AGCTGACCGG CATCCCGCCG GCGCCGCGGG GGATTCCGCA
GATCGAGGTC ACTTTCGACA TCGACGCCAA CGGCATTGTG CACGTCACCG CCAAGGACAA GGGCACCGGC AAGGAGAACA CGATCCGAAT
CCAGGAAGGC TCGGGCCTGT CCAAGGAAGA CATTGACCGC ATGATCAAGG ACGCCGAAGC GCACGCCGAG GAGGATCGCA AGCGTCGCGA
GGAGGCCGAT GTTCGTAATC AAGCCGAGAC ATTGGTCTAC CAGACGGAGA AGTTCGTCAA AGAACAGCGT GAGGCCGAGG GTGGTTCGAA
GTTCGTAATC AAGCCGAGAC ATTGGTCTAC CAGACGGAGA AGTTCGTCAA AGAACAGCGT GAGGCCGAGG GTGGTTCGAA GGTACCTGAA
GACACGCTGA ACAAGGTTGA TGCCGCGGTG GCGGAAGCGA AGGCGGCACT TGGCGGATCG GATATTTCGG CCATCAAGTC GGCGATGGAG
AAGCTGGGCC AGGAGTCGCA GGCTCTGGGG CAAGCGATCT ACGAAGCAGC TCAGGCTGCG TCACAGGCCA CTGGCGCTGC CCACCCCGGC
TCGGCTGATG AAAGCTTaag tttaaaccgc tgatcagcct cgactgtgcc ttctagttgc cagccatctg ttgtttgccc ctcccccgtg
ccttccttga ccctggaagg tgccactccc actgtccttt cctaataaaa tgaggaaatt gcatcgcatt gtctgagtag gtgtcattct
attctggggg gtggggtggg gσaggacagc aaggggggagg attgggaaga caatagcagg catgctgggg atgcggtggg ctctatggct
tctgaggcgg aaagaaccag ctggggctct aggggggtatc cccacgcgcc ctgtagcggc gcattaagcg cggcgggtgt ggtggttacg
cgcagcgtga ccgctacact tgccagcgcc ctagcgcccg ctcctttcgc tttcttccct tcctttctcg ccacgttcgc cggctttccc
cgtcaagctc taaatcgggg catcccttta gggttccgat ttagtgcttt acggcacctc gacσccaaaa aacttgatta gggtgatggt
tcaσgtagtg ggccatcgcc ctgatagacg gtttttcgcc ctttgacgtt ggagtccacg ttctttaata gtggactctt gttccaaact
ggaacaacac tcaaccctat ctcggtctat tcttttgatt tataagggat tttggggatt tcggcctatt ggttaaaaaa tgagctgatt
```

```
      taacaaaaat ttaacgcgaa ttaattctgt ggaatgtgtg tcagttaggg tgtggaaagt ccccaggctc cccaggcagg cagaagtatg
      caaagcatgc atctcaatta gtcagcaacc aggtgtggaa agtccccagg ctccccagca ggcagaagta tgcaaagcat gcatctoaat
      tagtcagcaa ccatagtccc gcccctaact ccgcccatcc cgcccctaac tccgcccagt tccgcccatt ctccgcccca tggctgacta
      attttttta tttatgcaga ggccgaggcc gcctctgcct ctgagctatt ccagaagtag tgaggaggct tttttggagg cctaggcttt
 5    tgcaaaaagc tcccgggagc ttgtatatcc attttcggat ctgatcaaga gacaggatga ggatcgtttc gcatgattga acaagatgga
      ttgcacgcag gttctccggc cgcttgggtg gagaggctat tcggctatga ctgggcacaa cagacaatcg gctgctctga tgccgccgtg
      ttccggctgt cagcgcaggg gcgcccggtt ctttttgtca agaccgacct gtccggtgcc ctgaatgaac tgcaggacga ggcagcgcgg
      ctatcgtggc tggccacgac gggcgttcct tgcgcagctg tgctcgacgt tgtcactgaa tgcaggacga ggcagcgcgg ctatcgtggc
      tggccacgac gggcgttcct tgcgcagctg tgctcgacgt tgtcactgaa gcgggaaggg actggctgct attgggcgaa gtgccggggc
10    aggatctcct gtcatctcac cttgctcctg ccgagaaagt atcσatcatg gctgatgcaa tgcggcggct gcatacgctt gatccggcta
      cctgcccatt cgaccaccaa gcgaaacatc gcatcgagcg agcacgtact cggatggaag ccggtcttgt cgatcaggat gatctggaσg
      aagagcatca ggggctcgcg ccagccgaac tgttcgccag gctcaaggcg cgcatgcccg acggcgagga tctcgtcgtg acccatggcg
      atgcctgctt gccgaatatc atggtggaaa atggccgctt ttctggattc atcgactgtg gccggctggg tgtggcggac cgctatcagg
      acatagcgtt ggctacccgt gatattgctg aagagcttgg cggcgaatgg gctgaccgct tcctcgtgct ttacggtatc gccgctccσg
15    attcgcagcg catcgccttc tatcgccttc ttgacgagtt cttctgagcg ggactctggg gttcgaaatg accgaccaag cgacgcccaa
      cctgccatca cgagatttcg attccaccgc cgccttctat gaaaggttgg gcttcggaat cgttttcσgg gacgccggct ggatgatcct
      ccagcgcggg gatctcatgc tggagttctt cgcccacccc aacttgttta ttgcagctta taatggttac aaataaagca atagcatcac
      aaatttcaca aataaagcat ttttttcact gcattctagt tgtggtttgt ccaaactcat caatgtatct tatcatgtct gtataccgtc
      gacctctagc tagagcttgg cgtaatσatg gtcatagctg tttcctgtgt gaaattgtta tccgctcaca attccacaca acatacgagc
20    cggaagcata aagtgtaaag cctggggtgc ctaatgagtg agctaactca cattaattgc gttgcgctca ctgcccgctt tccagtcggg
      aaacctgtcg tgccagctgc attaatgaat cggccaacgc gcggggagag gcggtttgcg tattgggcgc tcttccgctt cctcgctcac
      tgactcgctg cgctcggtcg ttcggctgcg gcgagcggta tcagctcact caaaggcggt aatacggtta tccacagaat caggggataa
      cgcaggaaag aacatgtgag caaaaggcca gcaaaaggcc aggaaccgta aaaaggccgc gttgctggcg catcacaaaa atcgacgctc
      aagtcagagg tggcgaaacc cgacaggact ataaagatac caggcgtttc cσcctggaag ctccctcgtg cgctctcctg ttccgaccct
25    gccgcttacc ggatacctgt ccgσctttct cccttcggga agcgtggcgc tttctcaatg ctcacgctgt aggtatctca gttcggtgta
      ggtcgttcgc tccaagctgg gctgtgtgca cgaacccccc gttcagcccg accgctgcgc cttatccggt aactatcgtσ ttgagtccaa
      ccσggtaaga cacgacttat cgccactggc agcagccact ggtaacagga ttagcagagc gaggtatgta ggcggtgcta cagagttctt
      gaagtggtgg cctaactacg gctacactag aaggacagta tttggtatct gcgctctgct gaagccagtt accttcggaa aaagagttgg
      tagσtcttga tccggcaaac aaaccaccgc tggtagcggt ggtttttttg tttgcaagca gcagattacg cgcagaaaaa aaggatctca
30    agaagatcct ttgatctttt ctacggggtc tgacgctcag tggaacgaaa actcacgtta agggattttg gtcatgagat tatcaaaaag
      gatcttcacc tagatccttt taaattaaaa atgaagtttt aaatcaatct aaagtatata tgagtaaact tggtctgaca gttaccaatg
      cttaatcagt gaggcaccta tctcagcgat ctgtctattt cgttcatcca tagttgcctg actccccgtc gtgtagataa ctacgatacg
      ggagggctta ccatctggcc ccagtgctgc aatgataccg cgagacccac gctcaccggc tccagattta tcagcaataa accagccagc
      cggaagggcc gagcgcagaa gtggtcctgc aactttatcc gcctccatcc agtctattaa ttgttgccgg gaagctagag taagtagttc
35    gccagttaat agtttgcgca acgttgttgc cattgctaca ggcatcgtgg tgtcacgctc gtcgtttggt atggcttcat tcagctccgg
      ttcccaacga tcaaggcgag ttacatgatc ccccatgttg tgcaaaaaag cggttagctc cttcggtcct ccgatcgttg tcagaagtaa
      gttggccgca gtgttatcac tcatggttat ggcagcactg cataattctc ttactgtcat gccatccgta agatgctttt ctgtgactgg
      tgagtactca accaagtcat tctgagaata gtgtatgcgg cgaccgagtt gctcttgccc ggcgtcaata cgggataata ccgcgccaca
      tagcagaact ttaaaagtgc tcatcattgg aaaacgttct tcggggcgaa aactctcaag gatcttaccg σtgttgagat ccagttcgat
40    gtaacccact cgtgcaccca actgatcttc agcatctttt actttcacca gcgtttctgg gtgagcaaaa acaggaaggc aaaatgccgc
      aaaaaaggga ataagggcga cacggaaatg ttgaatactc atactcttcc tttttcaata ttattgaagc atttatcagg gttattgtct
      catgagcgga tacatatttg aatgtattta gaaaaataaa caaatagggg ttccgcgcac atttccccga aaagtgccac ctgacgtc 7518
      I   10      I   20      I   30      I   40      I   50"     |   60      |   70      [   80      |     90     |
```

The nucleic acid sequence of plasmid construct pcDNA3-ETA(dH)/E7 (SEQ ID NO:37) is shown below. ETA(dH)/E7 is ligated into the EcoRI/BamHI sites of pcDNA3 vector. The nucleotides encoding ETA(dII)/E7 are shown in upper case and underscored. Plasmid sequence is lower case.

10	20	30	40	50	60	70	80	90
gacggatcgg	gagatctccc	gatcccctat	ggtcgactct	cagtacaatc	tgctctgatg	ccgcatagtt	aagccagtat	ctgctccctg
cttgtgtgtt	ggaggtcgct	gagtagtgcg	cgagcaaaat	ttaagctaca	acaaggcaag	gcttgaccga	caattgcatg	aagaatctgc
ttagggttag	gcgttttgcg	ctgcttcgcg	atgtacgggc	cagatatacg	cgttgacatt	gattattgac	tagttattaa	tagtaatcaa
ttacggggtc	attagttcat	agcccatata	tggagttccg	cgttacataa	cttacggtaa	atggcccgcc	tggctgaccg	cccaacgacc
cccgcccatt	gacgtcaata	atgacgtatg	ttcccatagt	aacgccaata	gggactttcc	attgacgtca	atgggtggac	tatttacggt
aaactgccca	cttggcagta	catcaagtgt	atcatatgcG	aagtacgccc	cctattgacg	tcaatgacgg	taaatggccc	gcctggcatt
atgcccagta	catgacctta	tgggactttc	ctacttggca	gtacatctac	gtattagtca	tcgctattac	catggtgatg	cggttttggc
agtacatcaa	tgggcgtgga	tagcggtttg	actcacgggg	atttccaagt	ctccacccca	ttgacgtcaa	tgggagtttg	ttttggcacc
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gtttaaacgg	gccctctaga	ctcgagcggc	cgccactgtg	ctggatatct	gcagaattcA	TGCGCCTGCA	CTTTCCCGAG	GGCGGCAGCC
TGGCCGCGCT	GACCGCGCAC	CAGGCTTGCC	ACCTGCCGCT	GGAGACTTTC	ACCCGTCATC	GCCAGCCGCG	CGGCTGGGAA	CAACTGGAGC
AGTGCGGCTA	TCCGGTGCAG	CGGCTGGTCG	CCCTCTACCT	GGCGGCGCGG	CTGTCGTGGA	ACCAGGTCGA	CCAGGTGATC	CGCAACGCCC
TGGCCAGCCC	CGGCAGCGGC	GGCGACCTGG	GCGAAGCGAT	CCGCGAGCAG	CCGGAGCAGG	CCCGTCTGGC	CCTGACCCTG	GCCGCCGCCG
AGAGCGAGCG	CTTCGTCCGG	CAGGGCACCG	GCAACGACGA	GGCCGGCGCG	GCCAACGCCG	ACGTGGTGAG	CCTGACCTGC	CCGGTCGCCG
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ACGTCAGCTT	CAGCACCCGC	GGCACGCAGA	ACGAATTCAT	GCATGGAGAT	ACACCTACAT	TGCATGAATA	TATGTTAGAT	TTGCAACCAG
AGACAACTGA	TCTCTACTGT	TATGAGCAAT	TAAATGACAG	CTCAGAGGAG	GAGGATGAAA	TAGATGGTCC	AGCTGGACAA	GCAGAACCGG
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acggtttttc	gccctttgac	gttggagtcc	acgttcttta	atagtggact	cttgttccaa	actggaacaa	cactcaaccc	tatctcggtc
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10	20	30	40	50	60	70	80	90
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agggttattg	tctcatgagc	ggatacatat	ttgaatgtat	ttagaaaaat	aaacaaatag	gggttccgcg	cacatttccc	cgaaaagtgc
cacctgacgt	c							6221
I 10	20	30	40	I 50	60	70	80	90~~

Calreticulin (CRT)

"Calreticulin" or "CRT" describes the well-characterized -46 kDa resident protein of the ER lumen that has lectin activity and participates in the folding and assembly of nascent glycoproteins. CRT acts as a "chaperone" polypeptide and a member of the MHC class I transporter TAP complex; CRT associates with TAPl and TAP2 transporters, tapasin, MHC Class I heavy chain polypeptide and β2 microglobulin to function in the loading of peptide epitopes onto nascent MHC class I molecules (Jorgensen, *Eur. J. Biochem.* 267:2945-54, 2002. The term "calreticulin" or "CRT" refers to polypeptides and nucleic acids molecules having substantial identity (defined herein) to the exemplary CRT sequences as described herein. A CRT polypeptide is a polypeptides comprising a sequence identical to or substantially identical (defined herein) to the amino acid sequence of CRT. An exemplary nucleotide and amino acid sequence for a CRT used in the present compositions and methods are presented below. The terms "calreticulin" or "CRT" encompass native proteins as well as recombinantly produced modified proteins that induce an immune response, including a CTL response. The terms "calreticulin" or "CRT" encompass homologues and allelic variants of CRT, including variants of native proteins constructed by *in vitro* techniques, and proteins isolated from natural sources. The CRT polypeptides of the invention, and sequences encoding them, also include fusion proteins comprising non-CRT sequences, particularly MHC class I-binding peptides; and also further comprising other domains, *e.g.,* epitope tags, enzyme cleavage recognition sequences, signal sequences, secretion signals and the like.

The term "endoplasmic reticulum chaperone polypeptide" as used herein means any polypeptide having substantially the same ER chaperone function as the exemplary chaperone proteins CRT, tapasin, ER60 or calnexin. Thus, the term includes all functional fragments or variants or mimics thereof. A polypeptide or peptide can be routinely screened for its activity as an ER chaperone using assays known in the art, such as that set forth in Example 1. While the invention is not limited by any particular mechanism of action, *in vivo* chaperones promote the correct folding and oligomerization of many glycoproteins in the ER, including the assembly of the MHC class I heterotrimeric molecule (heavy (H) chain, β2m, and peptide). They also retain incompletely assembled MHC class I heterotrimeric complexes in the ER (Hauri FEBS Lett. 476:32-37, 2000).

The sequences of CRT, including human CRT, are well known in the art (McCauliffe, /. *Clin. Invest.* §6:332-5, 1990; Burns, *Nature* 367:476-80, 1994; Coppolino, *Int. J. Biochem. Cell Biol.* 30:553-8, 2000). The nucleic acid sequence appears as GenBank Accession No. NM 004343 and is SEQ ID NO:38.

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1    gtccgtactg cagagccgct gccggagggt cgttttaaag ggccgcgttg ccgccccctc
61   ggcccgccat gctgctatcc gtgccgctgc tgctcggcct cctcggcctg gccgtcgccg
121  agcccgccgt ctacttcaag gagcagtttc tggacggaga cgggtggact tcccgctgga
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181   tcgaatccaa   acacaagtca   gattttggca   aattcgttct   cagttccggc   aagttctacg
241   gtgacgagga   gaaagataaa   ggtttgcaga   caagccagga   tgcacgcttt   tatgctctgt
301   cggccagttt   cgagcctttc   agcaacaaag   gccagacgct   ggtggtgcag   ttcacggtga
361   aacatgagca   gaacatcgac   tgtgggggcg   gctatgtgaa   gctgtttcct   aatagtttgg
421   accagacaga   catgcacgga   gactcagaat   acaacatcat   gtttggtccc   gacatctgtg
481   gccctggcac   caagaaggtt   catgtcatct   tcaactacaa   gggcaagaac   gtgctgatca
541   acaaggacat   ccgttgcaag   gatgatgagt   ttacacacct   gtacacactg   attgtgcggc
601   cagacaacac   ctatgaggtg   aagattgaca   acagccaggt   ggagtccggc   tccttggaag
661   acgattggga   cttcctgcca   cccaagaaga   taaaggatcc   tgatgcttca   aaaccggaag
721   actgggatga   gcgggccaag   atcgatgatc   ccacagactc   caagcctgag   gactgggaca
781   agcccgagca   tatccctgac   cctgatgcta   agaagcccga   ggactgggat   gaagagatgg
841   acggagagtg   ggaaccccca   gtgattcaga   accctgagta   caagggtgag   tggaagcccc
901   ggcagatcga   caacccagat   tacaagggca   cttggatcca   cccagaaatt   gacaacccca
961   agtattctcc   cgatcccagt   atctatgcct   atgataactt   tggcgtgctg   ggcctggacc
1021  tctggcaggt   caagtctggc   accatctttg   acaacttcct   catcaccaac   gatgaggcat
1081  acgctgagga   gtttggcaac   gagacgtggg   gcgtaacaaa   ggcagcagag   aaacaaatga
1141  aggacaaaca   ggacgaggag   cagaggctta   aggaggagga   agaagacaag   aaacgcaaag
1201  aggaggagga   ggcagaggac   aaggaggatg   atgaggacaa   agatgaggat   gaggaggatg
1261  aggaggacaa   ggaggaagat   gaggaggaag   atgtccccgg   ccaggccaag   gacgagctgt
1321  agagaggcct   gcctccaggg   ctggactgag   gcctgagcgc   tcctgccgca   gagcttgccg
1381  cgccaaataa   tgtctctgtg   agactcgaga   actttcattt   ttttccaggc   tggttcggat
1441  ttggggtgga   ttttggtttt   gttcccctcc   tccactctcc   cccaccccct   ccccgccctt
1501  ttttttttttt  tttttaaact   ggtattttat   cctttgattc   tccttcagcc   ctcacccctg
1561  gttctcatct   ttcttgatca   acatcttttc   ttgcctctgt   gccccttctc   tcatctctta
1621  gctcccctcc   aacctggggg   gcagtggtgt   ggagaagcca   caggcctgag   atttcatctg
1681  ctctccttcc   tggagcccag   aggagggcag   cagaaggggg   tggtgtctcc   aaccccccag
1741  cactgaggaa   gaacggggct   cttctcattt   caccctccc   tttctccct   gccccccagga
1801  ctgggccact   tctgggtggg   gcagtgggtc   ccagattggc   tcacactgag   aatgtaagaa
1861  ctacaaacaa   aatttctatt   aaattaaatt   ttgtgtctc                1899
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Human CRT protein (GenBank Accession No. NM 004343), (SEQ ID NO:39) is shown below:

```
1     MLLSVPLLLG   LLGLAVAEPA   VYFKEQFLDG   DGWTSRWIES   KHKSDFGKFV   LSSGKFYGDE
61    EKDKGLQTSQ   DARFYALSAS   FEPFSNKGQT   LVVQFTVKHE   QNIDCGGGYV   KLFPNSLDQT
121   DMHGDSEYNI   MFGPDICGPG   TKKVHVIFNY   KGKNVLINKD   IRCKDDEFTH   LYTLIVRPDN
181   TYEVKIDNSQ   VESGSLEDDW   DFLPPKKIKD   PDASKPEDWD   ERAKIDDPTD   SKPEDWDKPE
241   HIPDPDAKKP   EDWDEEMDGE   WEPPVIQNPE   YKGEWKPRQI   DNPDYKGTWI   HPEIDNPEYS
301   PDPSIYAYDN   FGVLGLDLWQ   VKSGTIFDNF   LITNDEAYAE   EFGNETWGVT   KAAEKQMKDK
361   QDEEQRLKEE   EEDKKRKEEE   EAEDKEDDED   KDEDEEDEED   KEEDEEEDVP   GQAKDEL     417
```

For the generation of plasmid encoding the full length of rabbit calreticulin (there is more than 90% homology between rabbit, human, mouse, and rat calreticulin), pcDNA3-CRT, the DNA fragment encoding this protein was first amplified with PCR using conditions as described in Chen, *Cancer Res.*, 2000, *supra,* using rabbit calreticulin cDNA template (Michalak, *Biochem J. 344 Pt* 2:281-292, 1999), provided by Dr. Marek Michalak, University of Alberta, Edmonton, Canada, and a set of the following primers:

```
5'-ccggtctagaatgctgctccctgtgccgct-3     ' (SEQ ID NO:40) and
5'-ccggagatctcagctcgtccttggcctggc-3'      (SEQIDNO:41)
```

The amplified product was then digested with the restriction digest enzymes XbaI and BamHI and further cloned into the XbaI and BamHI cloning sites of pcDNA3 vector (Invitrogen, Carlsbad, CA). For the generation of pcDNA3-CRT/E7, the E7 DNA was amplified by PCR using pcDNA3-E7 as a DNA template and a set of primers:

5'-ggggaattcatggagatacaccta-3' (SEQ ED NO:42) and
5'-ggtggatccttgagaacagatgg-3' (SEQ ID NO:43).

The amplified E7 DNA fragment was then digested with BamHI and further cloned into the BamHI cloning sites of pcDNA3-CRT vector. The orientation and accuracy of these constructs was confirmed by DNA sequencing.

Plasmid DNA with CRT, E7 or CRT/E7 gene insert and the "empty" plasmid vector were transfected into subcloning-efficient DH5™ cells (Life Technologies, USA). The DNA was then amplified and purified using double CsCl purification (BioServe Biotechnologies, Laurel, MD). The integrity of plasmid DNA and the absence of *E. coli* DNA or RNA were verified by agarose gel electrophoresis, and the presence of the inserted E7 fragment was confirmed by restriction enzyme digestion and gel electrophoresis.

The present inventors and their colleagues have found that DNA vaccines encoding CRT linked either to E6 or to E7 both of generate significant antitumor effects against E6- and E7-expressing tumors, respectively. Moreover, simultaneous vaccination with both CRT/E6 and CRT/E7 DNA vaccines generated significant E6- and E7-specific T-cell immune responses and significantly better therapeutic antitumor effects against E6- and E7-expressing tumors than vaccination with either CRT/E6 DNA or CRT/E7 DNA alone.

The three domains of CRT also produce E7-specific antitumor immunity and antiangiogenic effects (Cheng WF *et at, Vaccine.* 23:3864-74, 2005). DNA vaccines encoding each of N, P, and C domains of CRT linked to E7 antigen produced significant stimulation of E7-specific $CD8^+$ T cell precursors and antitumor effects against E7-expressing tumors. The N domain of CRT also showed antiangiogenic properties that might have contributed to the antitumor effect. Thus, the present invention includes DNA immunogens in which the IPP is the N, P, or C domain of CRT.

The nucleotide sequence of plasmid *pNGVL4a-CRT/E7(detox)* (SEQ DD NO:44) is shown below. The sequence is annotated to show plasmid-derived sequences(lower case), CRT-derived (bold, upper case) and HPV-E7-derived (detoxified by two amino acid substitutions as described above(upper case, italicized, underlined) sequences.

pNGVL4a-CRT/E7(detox) CSEO ID NO:44)

gctccgcccc cctgacgagc atcacaaaaa tcgacgctca agtcagaggt ggcgaaaccc gacaggacta taaagatacc aggcgtttcc
ccctggaagc tccctcgtgc gctctcctgt tccgaccctg ccgcttaccg gatacctgtc cgcctttctc ccttcgggaa gcgtggcgct
ttctcatagc tcacgctgta ggtatctcag ttcggtgtag gtcgttcgct ccaagctggg ctgtgtgcac gaaccccccg ttcagcccga
ccgctgcgcc ttatccggta actatcgtct tgagtccaac ccggtaagac acgacttatc gccactggca gcagccactg gtaacaggat
tagcagagcg aggtatgtag gcggtgctac agagttcttg aagtggtggc ctaactacgg ctacactaga agaacagtat ttggtatctg
cgctctgctg aagccagtta ccttcggaaa aagagttggt agctcttgat ccggcaaaca aaccaccgct ggtagcggtg gttttttgt
ttgcaagcag cagattacgc gcagaaaaaa aggatctcaa gaagatcctt tgatcttttc tacggggtct gacgctcagt ggaacgaaaa
ctcacgttaa gggattttgg tcatgagatt atcaaaaagg atcttcacct agatccttt aaattaaaaa tgaagtttta aatcaatcta
aagtatatat gagtaaactt ggtctgacag ttaccaatgc ttaatcagtg aggcacctat ctcagcgatc tgtctatttc gttcatccat
agttgcctga ctcgggggggg gggggcgctg aggtctgcct cgtgaagaag gtgttgctga ctcataccag ggcaacgttg ttgccattgc
tacaggcatc gtggtgtcac gctcgtcgtt tggtatggct tcattcagct ccggttccca acgatcaagg cgagttacat gatcccccat
gttgtgcaaa aaagcggtta gctccttcgg tcctccgatc gttgtcagaa gtaagttggc cgcagtgtta tcactcatgg ttatggcagc
actgcataat tctcttactg tcatgccatc cgtaagatgc ttttctgtga ctggtgagta ctcaaccaag tcattctgag aatagtgtat
gcggcgaccg agttgctctt gcccggcgtc aatacgggat aataccgcgc cacatagcag aactttaaaa gtgctcatca ttggaaaacg
ttcttcgggg cgaaaactct caaggatctt accgctgttg agatccagtt cgatgtaacc cactcgtgca cctgaatcgc cccatcatcc
agccagaaag tgagggagcc acggttgatg agagctttgt tgtaggtgga ccagttggtg attttgaact tttgctttgc cacggaacgg
tctgcgttgt cgggaagatg cgtgatctga tccttcaact cagcaaaagt tcgatttatt caacaaagcc gccgtcccgt caagtcagcg
taatgctctg ccagtgttac aaccaattaa ccaattctga ttagaaaaac tcatcgagca tcaaatgaaa ctgcaattta ttcatatcag
gattatcaat accatatttt tgaaaaagcc gtttctgtaa tgaaggagaa aactcaccga ggcagttcca taggatggca agatcctggt
atcggtctgc gattccgact cgtccaacat caatacaacc tattaatttc ccctcgtcaa aaataaggtt atcaagtgag aaatcaccat
gagtgacgac tgaatccggt gagaatggca aaagcttatg catttctttc cagacttgtt caacaggcca gccattacgc tcgtcatcaa
aatcactcgc atcaaccaaa ccgttattca ttcgtgattg cgcctgagcg agacgaaata cgcgatcgct gttaaaagga caattacaaa
caggaatcga atgcaaccgg cgcaggaaca ctgccagcgc atcaacaata ttttcacctg aatcaggata ttcttctaat acctggaatg
ctgttttccc ggggatcgca gtggtgagta accatgcatc atcaggagta cggataaaat gcttgatggt cggaagaggc ataaattccg
tcagccagtt tagtctgacc atctcatctg taacatcatt ggcaacgcta cctttgccat gtttcagaaa caactctggc gcatcgggct
tcccatacaa tcgatagatt gtcgcacctg attgcccgac attatcgcga gcccatttat acccatataa atcagcatcc atgttggaat
ttaatcgcgg cctcgagcaa gacgtttccc gttgaatatg gctcataaca ccccttgtat tactgtttat gtaagcagac agttttattg
ttcatgatga tatattttta tcttgtgcaa tgtaacatca gagattttga gacacaacgt ggctttcccc ccccccccat tattgaagca
tttatcaggg ttattgtctc atgagcggat acatatttga atgtatttag aaaaataaac aaatagggggt tccgcgcaca tttccccgaa
aagtgccacc tgacgtctaa gaaaccatta ttatcatgac attaacctat aaaaataggc gtatcacgag gccctttcgt ctcgcgcgtt
tcggtgatga cggtgaaaac ctctgacaca tgcagctccc ggagacggtc acagcttgtc tgtaagcgga tgccgggagc agacaagccc
gtcagggcgc gtcagcgggt gttggcgggt gtcggggctg gcttaactat gcggcatcag agcagattgt actgagagtg caccatatgc
ggtgtgaaat accgcacaga tgcgtaagga gaaaataccg catcagattg gctattggcc attgcatacg ttgtatccat atcataatat
gtacatttat attggctcat gtccaacatt accgccatgt tgacattgat tattgactag ttattaatag taatcaatta cggggtcatt
agttcatagc ccatatatgg agttccgcgt tacataactt acggtaaatg gcccgcctgg ctgaccgccc aacgaccccc gcccattgac
gtcaataatg acgtatgttc ccatagtaac gccaataggg actttccatt gacgtcaatg ggtggagtat ttacggtaaa ctgcccactt

ggcagtacat caagtgtatc atatgccaag tacgcccccc attgacgtca atgacggtaa atggcccgcc tggcattatg cccagtacat
gaccttatgg gactttccta cttggcagta catctacgta ttagtcatcg ctattaccat ggtgatgcgg ttttggcagt acatcaatgg
gcgtggatag cggtttgact cacggggatt tccaagtctc caccccattg acgtcaatgg gagtttgttt tggcaccaaa atcaacggga
ctttccaaaa tgtcgtaaca actccgcccc attgacgcaa atgggcggta ggcgtgtacg gtgggaggtc tatataagca gagctcgttt
agtgaaccgt cagatcgcct ggagacgcca tccacgctgt tttgacctcc atagaagaca ccgggaccga tccagcctcc gcggccggga
acggtgcatt ggaacgcgga ttccccgtgc caagagtgac gtaagtaccg cctatagact ctataggcac acccctttgg ctcttatgca
tgctatactg tttttggctt ggggcctata cacccccgct tccttatgct ataggtgatg gtatagctta gcctataggt gtgggttatt
gaccattatt gaccactcca acggtggagg gcagtgtagt ctgagcagta ctcgttgctg ccgcgcgcgc caccagacat aatagctgac
agactaacag actgttcctt tccatgggtc ttttctgcag tcaccgtcgt cgacATGCTG CTATCCGTGC CGCTGCTGCT CGGCCTCCTC
GGCCTGGCCG TCGCCGAGCC TGCCGTCTAC TTCAAGGAGC AGTTTCTGGA CGGGGACGGG TGGACTTCCC GCTGGATCGA ATCCAAACAC
AAGTCAGATT TTGGCAAATT CGTTCTCAGT TCCGGCAAGT TCTACGGTGA CGAGGAGAAA GATAAAGGTT TGCAGACAAG CCAGGATGCA
CGCTTTTATG CTCTGTCGGC CAGTTTCGAG CCTTTCAGCA ACAAAGGCCA GACGCTGGTG GTGCAGTTCA CGGTGAAACA TGAGCAGAAC
ATCGACTGTG GGGGCGGCTA TGTGAAGCTG TTTCCTAATA GTTTGGACCA GACAGACATG CACGGAGACT CAGAATACAA CATCATGTTT
GGTCCCGACA TCTGTGGCCC TGGCACCAAG AAGGTTCATG TCATCTTCAA CTACAAGGGC AAGAACGTGC TGATCAACAA GGACATCCGT
TGCAAGGATG ATGAGTTTAC ACACCTGTAC ACACTGATTG TGCGGCCAGA CAACACCTAT GAGGTGAAGA TTGACAACAG CCAGGTGGAG
TCCGGCTCCT TGGAAGACGA TTGGGACTTC CTGCCACCCA AGAAGATAAA GGATCCTGAT GCTTCAAAAC CGGAAGACTG GGATGAGCGG
GCCAAGATCG ATGATCCCAC AGACTCCAAG CCTGAGGACT GGGACAAGCC CGAGCATATC CCTGACCCTG ATGCTAAGAA GCCCGAGGAC
TGGGATGAAG AGATGGACGG AGAGTGGGAA CCCCCAGTGA TTCAGAACCC TGAGTACAAG GGTGAGTGGA AGCCCCGGCA GATCGACAAC
CCAGATTACA AGGGCACTTG GATCCACCCA GAAATTGACA ACCCCGAGTA TTCTCCCGAT CCCAGTATCT ATGCCTATGA TAACTTTGGC
GTGCTGGGCC TGGACCTCTG GCAGGTCAAG TCTGGCACCA TCTTTGACAA CTTCCTCATC ACCAACGATG AGGCATACGC TGAGGAGTTT
GGCAACGAGA CGTGGGGCGT AACAAAGGCA GCAGAGAAAC AAATGAAGGA CAAACAGGAC GAGGAGCAGA GGCTTAAGGA GGAGGAAGAA
GACAAGAAAC GCAAAGAGGA GGAGGAGGCA GAGGACAAGG AGGATGATGA GGACAAAGAT GAGGATGAGG AGGATGAGGA GGACAAGGAG
GAAGATGAGG AGGAAGATGT CCCCGGCCAG GCCAAGGACG AGCTGgaatt *cATGCATGGA GATACACCTA CATTGCATGA ATATATGTTA*
GATTTGCAAC CAGAGACAAC TGATCTCTAC GGTTATGGGC AATTAAATGA CAGCTCAGAG GAGGAGGATG AAATAGATGG TCCAGCTGGA
CAAGCAGAAC CGGACAGAGC CCATTACAAT ATTGTAACCT TTTGTTGCAA GTGTGACTCT ACGCTTCGGT TGTGCGTACA AAGCACACAC
GTAGACATTC GTACTTTGGA AGACCTGTTA ATGGGCACAC TAGGAATTGT GTGCCCCATC TGTTCTCAGA AACCATAA gg atccagatct
ttttccctct gccaaaaatt atggggacat catgaagccc cttgagcatc tgacttctgg ctaataaagg aaatttattt tcattgcaat
agtgtgttgg aatttttttgt gtctctcact cggaaggaca tatgggaggg caaatcattt aaaacatcag aatgagtatt tggtttagag
tttggcaaca tatgcccatt cttccgcttc ctcgctcact gactcgctgc gctcggtcgt tcggctgcgg cgagcggtat cagctcactc
aaaggcggta atacggttat ccacagaatc aggggataac gcaggaaaga acatgtgagc aaaaggccag caaaaggcca ggaaccgtaa
aaaggccgcg ttgctggcgt ttttccatag 5970

GENERAL RECOMBINANT DNA METHODS

Basic texts disclosing general methods of molecular biology, all of which are incorporated by reference, include: Sambrook, J *et at, Molecular Cloning: A Laboratory Manual,* 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989; Ausubel, FM *et at Current Protocols in Molecular Biology,* Vol. 2, Wiley-Interscience, New York, (current edition); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); Glover, DM, ed, *DNA Cloning: A Practical Approach,* vol. I & π, IRL Press, 1985; Albers, B. *et at, Molecular Biology of the Cell,* 2nd Ed., Garland Publishing, Inc., New York, NY (1989); Watson, JD *et at, Recombinant DNA,* 2nd Ed., Scientific American Books, New York, 1992; and Old, RW *et at, Principles of Gene Manipulation: An Introduction to Genetic Engineering,* 2nd Ed., University of California Press, Berkeley, CA (1981).

Techniques for the manipulation of nucleic acids, such as, *e.g.,* generating mutations in sequences, subcloning, labeling probes, sequencing, hybridization and the like are well described in the scientific and patent literature. See, *e.g.,* Sambrook, ed., MOLECULAR CLONING: ALABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997); LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I. Tijssen, ed. Elsevier, N.Y. (1993).

Nucleic acids, vectors, capsids, polypeptides, and the like can be analyzed and quantified by any of a number of general means well known to those of skill in the art. These include, *e.g.,* analytical biochemical methods such as NMR, spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography, various immunological methods, *e.g.* fluid or gel precipitin reactions, immunodiffusion, immuno-electrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immunofluorescence assays, Southern analysis, Northern analysis, dot-blot analysis, gel electrophoresis *{e.g.,* SDS-PAGE), RT-PCR, quantitative PCR, other nucleic acid or target or signal amplification methods, radiolabeling, scintillation counting, and affinity chromatography.

Amplification of Nucleic Acids

Oligonucleotide primers can be used to amplify nucleic acids to generate fusion protein coding sequences used to practice the invention, to monitor levels of vaccine after *in vivo* administration *{e.g.,* levels of a plasmid or virus), to confirm the presence and phenotype of activated CTLs, and the like. The skilled artisan can select and design suitable oligonucleotide amplification primers using known sequences. Amplification methods are also well known in the art, and include, *e.g.,* polymerase chain

reaction, PCR *(PCR Protocols, A Guide to Methods and Applications,* ed. Innis, Academic Press, N.Y. (1990) and *PCR Strategies* (1995), ed. Innis, Academic Press, Inc., N.Y., ligase chain reaction (LCR) (Wu (1989) *Genomics* 4:560; Landegren (1988) *Science 242:1077;* Barringer (1990) Gene 89:117); transcription amplification (Kwoh (1989) *Proc. Natl. Acad. ScL USA 86:1173);* and, self-sustained sequence replication (Guatelli (1990) *Proc. Natl. Acad. ScL USA 87:* 1874); Qβ replicase amplification (Smith (1997) *J. Clin. Microbiol.* 35:1477-1491; Burg (1996) *MoI. Cell. Probes* 20:257-271) and other RNA polymerase mediated techniques (NASBA, Cangene, Mississauga, Ontario; Berger (1987) *Methods Enzymol. 152:307-316;* U.S. Pats No. 4,683,195 and 4,683,202; Sooknanan (1995) *Biotechnology* 73:563-564).

Unless otherwise indicated, a particular nucleic acid sequence is intended to encompasses conservative substitution variants thereof *(e.g.,* degenerate codon substitutions) and a complementary sequence. The term "nucleic acid" is synonymous with "polynucleotide" and is intended to include a gene, a cDNA molecule, an mRNA molecule, as well as a fragment of any of these such as an oligonucleotide, and further, equivalents thereof (explained more fully below). Sizes of nucleic acids are stated either as kilobases (kb) or base pairs (bp). These are estimates derived from agarose or polyacrylamide gel electrophoresis (PAGE), from nucleic acid sequences which are determined by the user or published. Protein size is stated as molecular mass in kilodaltons (kDa) or as length (number of amino acid residues). Protein size is estimated from PAGE, from sequencing, from presumptive amino acid sequences based on the coding nucleic acid sequence or from published amino acid sequences.

Specifically, cDNA molecules encoding the amino acid sequence corresponding to the fusion polypeptide of the present invention or fragments or derivatives thereof can be synthesized by the polymerase chain reaction (PCR) (see, for example, U.S. 4,683,202) using primers derived the sequence of the protein disclosed herein. These cDNA sequences can then be assembled into a eukaryotic or prokaryotic expression vector and the resulting vector can be used to direct the synthesis of the fusion polypeptide or its fragment or derivative by appropriate host cells, for example COS or CHO cells.

This invention includes isolated nucleic acids having a nucleotide sequence encoding the novel fusion polypeptides that comprise a translocation polypeptide and an antigen, fragments thereof or equivalents thereof. The term nucleic acid as used herein is intended to include such fragments or equivalents. The nucleic acid sequences of this invention can be DNA or RNA.

A cDNA nucleotide sequence the fusion polypeptide can be obtained by isolating total mRNA from an appropriate cell line. Double stranded cDNA is prepared from total mRNA. cDNA can be inserted into a suitable plasmid, bacteriophage or viral vector using any one of a number of known techniques.

In reference to a nucleotide sequence, the term "equivalent" is intended to include sequences encoding structurally homologous and/or a functionally equivalent proteins. For example, a natural polymorphism in a nucleotide sequence encoding an anti-apoptotic polypeptide according to the present invention (especially at the third base of a codon) may be manifest as "silent" mutations which do not change the amino acid sequence. Furthermore, there may be one or more naturally occurring isoforms or related, immunologically cross-reactive family members of these proteins. Such isoforms or family members are defined as proteins that share function amino acid sequence similarity to the reference polypeptide.

Fragment of Nucleic Acid

A fragment of the nucleic acid sequence is defined as a nucleotide sequence having fewer nucleotides than the nucleotide sequence encoding the full length translocation polypeptide, antigenic polypeptide or the fusion thereof. This invention includes such nucleic acid fragments that encode polypeptides which retain (1) the ability of the fusion polypeptide to induce increases in frequency or reactivity of T cells, preferably CD8+ T cells, that are specific for the antigen part of the fusion polypeptide.

For example, a nucleic acid fragment as intended herein encodes an antigen or an IPP that that retains the ability to improve the immunogenicity of an antigen vaccine when administered as a chimeric DNA with antigen-encoding sequence, or when co-administered therewith.

Generally, the nucleic acid sequence encoding a fragment of an anti-apoptotic polypeptide comprises of nucleotides from the sequence encoding the mature protein (or an active fragment thereof).

Nucleic acid sequences of this invention may also include linker sequences, natural or modified restriction endonuclease sites and other sequences that are useful for manipulations related to cloning, expression or purification of encoded protein or fragments. These and other modifications of nucleic acid sequences are described herein or are well-known in the art.

The techniques for assembling and expressing DNA coding sequences for translocation types of proteins, and DNA coding sequences for antigenic polypeptides, include synthesis of oligonucleotides, PCR, transforming cells, constructing vectors, expression systems, and the like; these are well-established in the art such that those of ordinary skill are familiar with standard resource materials, specific conditions and procedures.

EXPRESSION VECTORS AND HOST CELLS

This invention includes an expression vector comprising a nucleic acid sequence encoding (a) an antigen, optionally linked to (b) an IPP or (c) an siRNA operably linked to at least one regulatory sequence, which includes a promoter that is expressable in a eukaryotic cell, preferably in a mammalian cells, more preferably in a human cell.

The term "expression vector" or "expression cassette" as used herein refers to a nucleotide sequence which is capable of affecting expression of a protein coding sequence in a host compatible with such sequences. Expression cassettes include at least a promoter operably linked with the polypeptide coding sequence; and, optionally, with other sequences, *e.g.*, transcription termination signals. Additional factors necessary or helpful in effecting expression may also be included, *e.g.*, enhancers.

"Operably linked" means that the coding sequence is linked to a regulatory sequence in a manner that allows expression of the coding sequence. Known regulatory sequences are selected to direct expression of the desired protein in an appropriate host cell. Accordingly, the term "regulatory sequence" includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in, for example, Goeddel, *Gene Expression Technology. Methods in Enzymology,* vol. 185, Academic Press, San Diego, Calif. (1990)).

Thus, expression cassettes include plasmids, recombinant viruses, any form of a recombinant "naked DNA" vector, and the like. A "vector" comprises a nucleic acid which can infect, transfect, transiently or permanently transduce a cell. It will be recognized that a vector can be a naked nucleic acid, or a nucleic acid complexed with protein or lipid. The vector optionally comprises viral or bacterial nucleic acids and/or proteins, and/or membranes *(e.g.,* a cell membrane, a viral lipid envelope, *etc.).* Vectors include replicons *(e.g.,* RNA replicons), bacteriophages) to which fragments of DNA may be attached and become replicated. Vectors thus include, but are not limited to RNA, autonomous self-replicating circular or linear DNA or RNA, *e.g.*, plasmids, viruses, and the like (U.S. Patent No. 5,217,879), and includes both the expression and nonexpression plasmids. Where a recombinant cell or culture is described as hosting an "expression vector" this includes both extrachromosomal circular and linear DNA and DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome.

Those skilled in the art appreciate that the particular design of an expression vector of this invention depends on considerations such as the host cell to be transfected and/or the type of protein to be expressed.

The present expression vectors comprise the full range of nucleic acid molecules encoding the various embodiments of the fusion polypeptide and its functional derivatives (defined herein) including polypeptide fragments, variants, *etc.,* as well as those encoding siRNA or other siNAs of the present invention.

Such expression vectors are used to transfect host cells *(in vitro, ex vivo* or *in vivo)* for expression of the DNA and production of the encoded proteins which include fusion proteins or

peptides. It will be understood that a genetically modified cell expressing the fusion polypeptide may transiently express the exogenous DNA for a time sufficient for the cell to be useful for its stated purpose.

The present in invention provides methods for producing the fusion polypeptides, fragments and derivatives. For example, a host cell transfected with a nucleic acid vector that encodes the fusion polypeptide or an siRNA is cultured under appropriate conditions to allow expression of the polypeptide or siRNA.

Host cells may also be transfected with one or more expression vectors that singly or in combination comprise (a) DNA encoding at least a portion of the fusion polypeptide and (b) DNA encoding at least a portion of a second protein, preferably an antigen, or (c) DNA encoding an siRNA, so that the host cells produce yet further fusion polypeptides or siRNAs

A culture typically includes host cells, appropriate growth media and other byproducts. Suitable culture media are well known in the art. The fusion polypeptide can be isolated from medium or cell lysates using conventional techniques for purifying proteins and peptides, including ammonium sulfate precipitation, fractionation column chromatography (e.g. ion exchange, gel filtration, affinity chromatography, *etc.)* and/or electrophoresis (see generally, "Enzyme Purification and Related Techniques", *Meth Enzymol,* 22:233-577 (1971)). Once purified, partially or to homogeneity, the recombinant polypeptides or siRNAs of the invention can be utilized in pharmaceutical compositions as described in more detail herein.

The term "isolated" as used herein, when referring to a molecule or composition, such as a translocation polypeptide or a nucleic acid coding therefor, means that the molecule or composition is separated from at least one other compound (protein, other nucleic acid, *etc.)* or from other contaminants with which it is natively associated or becomes associated during processing. An isolated composition can also be substantially pure. An isolated composition can be in a homogeneous state and can be dry or in aqueous solution. Purity and homogeneity can be determined, for example, using analytical chemical techniques such as polyacrylamide gel electrophoresis (PAGE) or high performance liquid chromatography (HPLC). Even where a protein has been isolated so as to appear as a homogenous or dominant band in a gel pattern, there are trace contaminants which co-purify with it.

Host cells transformed or transfected to express the fusion polypeptide or a homologue or functional derivative thereof are within the scope of the invention. For example, the fusion polypeptide may be expressed in yeast, or mammalian cells such as Chinese hamster ovary cells (CHO) or, preferably human cells. Preferred cells for expression of the siRNA of the present invention are APCs most preferably, DCs. Other suitable host cells are known to those skilled in the art.

Expression in eukaryotic cells leads to partial or complete glycosylation and/or formation of relevant inter- or intra-chain disulfide bonds of the recombinant protein.

Although preferred vectors are described in the Examples, other examples of expression vectors are provided here. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSecl (Baldari *et al, EMBO J.* 6:229-34, 1987), pMFa (Kurjan *et al, Cell* 30:933-43, 1982), pJRY88 (Schultz *et al, Gene* 54:113-23, 1987), and pYES2 (Invitrogen Corp.). Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith *et al., MoI. Cell Biol.* 3:2156-65, 1983) and the pVL series (Lucklow, VA *et al, Virology* 170:31-9, 1989). Generally, COS cells (Gluzman, Y., *Cell* 23:175-82, 1981) are used in conjunction with such vectors as pCDM 8 (Aruffo A *et al, supra,* for transient amplification/expression in mammalian cells, while CHO *(dhfr-negative* CHO) cells are used with vectors such as pMT2PC (Kaufman *et al. EMBO J.* 6:187-95, 1987) for stable amplification/expression in mammalian cells. The NSO myeloma cell line (a glutamine synthetase expression system.) is available from Celltech Ltd.

Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase, maltose E binding protein, or protein A, respectively, to the target recombinant protein. Inducible non-fusion expression vectors include pTrc (Amann *et al, Gene* 69:301-15, 1988) and pET 1Id (Studier *et al, Gene Expression Technology: Meth Enzymol* i §5:60-89, Academic Press, 1990).

Vector Construction

Construction of suitable vectors comprising the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and re-ligated in the form desired. The sequences of several preferred plasmid vectors, with and without inserted coding sequences, have been disclosed above.

The DNA sequences which form the vectors are available from a number of sources. Backbone vectors and control systems are generally found on available "host" vectors which are used for the bulk of the sequences in construction. For the pertinent coding sequence, initial construction may be, and usually is, a matter of retrieving the appropriate sequences from cDNA or genomic DNA libraries. However, once the sequence is disclosed it is possible to synthesize the entire gene sequence *in vitro* starting from the individual nucleotide derivatives. The entire gene sequence for genes of sizeable

length, *e.g.,* 500-1000 bp may be prepared by synthesizing individual overlapping complementary oligonucleotides and filling in single stranded nonoverlapping portions using DNA polymerase in the presence of the deoxyribonucleotide triphosphates. This approach has been used successfully in the construction of several genes of known sequence. See, for example, Edge, MD, *Nature* 292:756, 1981; Nambair, KP, *et al, Science* 223:1299, 1984; Jay, E, *J Biol Chem* 259:6311, 1984).

Synthetic oligonucleotides are prepared by either the phosphotriester method as described by references cited above or the phosphoramidite method (Beaucage, SL *et al, Tet Lett* 22:1859, 1981; Matteucci, MD *et al, J Am Chem Soc 103:3185,* 1981) and can be prepared using commercially available automated oligonucleotide synthesizers. Kinase treatment of single strands prior to annealing or for labeling is by conventional methods.

Once the components of the desired vectors are thus available, they can be excised and ligated using standard restriction and ligation procedures. Site-specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, *e.g.,* New England Biolabs, Product Catalog. A general description of size separations is found in *Methods in Enzymology* (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of *E. coli* DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using conventional methods and conditions. Ligations are performed using conventional methods. In vector construction employing "vector fragments", the fragment is commonly treated with bacterial or mammalian alkaline phosphatase to remove the 5' phosphate and prevent self-ligation. Alternatively, re-ligation can be prevented in vectors which have been double digested by additional restriction enzyme and separation of the unwanted fragments.

Any of a number of methods are used to introduce mutations into the coding sequence to generate the variants of the invention. These mutations include simple deletions or insertions, systematic deletions, insertions or substitutions of clusters of bases or substitutions of single bases.

For example, modifications of DNA sequences are created by site-directed mutagenesis, a well-known technique for which protocols and reagents are commercially available (Zoller, MJ *et al, Nucleic Acids Res* 10:6487-500, 1982; Adelman, JP *et al, DNA* 2:183-193, 1983). Using conventional methods, transformants are selected based on the presence of the ampicillin-, tetracycline-, or other antibiotic resistance gene (or other selectable marker) depending on the mode of plasmid construction. Plasmids are then prepared from the transformants with optional chloramphenicol amplification (Clewell, DB *et al, Proc Natl Acad Sci USA 62:1159,* 1969); Clewell, DB, *J Bacteriol 110:661,* 1969)). Several mini DNA preps are commonly used. See, *e.g., Anal Biochem 114:193-7,* 1981; *Nucleic Acids Res* 7:1513-23,

1979). The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy nucleotide method *of Sm@sτ (Proc Natl Acad Sd USA 74:5463,* 1977'; Messing, *et al, Nucleic Adds Res* 9:309, 1981), or by the method of Maxam *et al, Meth Enzymology 65:499,* 1980.

Vector DNA can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming host cells can be found in Sambrook *et al. supra* and other standard texts. In fusion expression vectors, a proteolytic cleavage site may be introduced at the junction of two sequences (such as a reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein). Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Promoters and Enhancers

A promoter region of a DNA or RNA molecule binds RNA polymerase and promotes the transcription of an "operably linked" nucleic acid sequence. As used herein, a "promoter sequence" is the nucleotide sequence of the promoter which is found on that strand of the DNA or RNA which is transcribed by the RNA polymerase. Two sequences of a nucleic acid molecule, such as a promoter and a coding sequence, are "operably linked" when they are linked to each other in a manner which permits both sequences to be transcribed onto the same RNA transcript or permits an RNA transcript begun in one sequence to be extended into the second sequence. Thus, two sequences, such as a promoter sequence and a coding sequence of DNA or RNA are operably linked if transcription commencing in the promoter sequence will produce an RNA transcript of the operably linked coding sequence. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another in the linear sequence.

The preferred promoter sequences of the present invention must be operable in mammalian cells and may be either eukaryotic or viral promoters. Although preferred promoters are described in the Examples, other useful promoters and regulatory elements are discussed below. Suitable promoters may be inducible, repressible or constitutive. A "constitutive" promoter is one which is active under most conditions encountered in the cell's environmental and throughout development. An "inducible" promoter is one which is under environmental or developmental regulation. A "tissue specific" promoter is active in certain tissue types of an organism. An example of a constitutive promoter is the viral promoter MSV-LTR, which is efficient and active in a variety of cell types, and, in contrast to most other promoters, has the same enhancing activity in arrested and growing cells. Other preferred viral promoters include that present in the CMV-LTR (from cytomegalovirus) (Bashart, M. *et al, Cell 41:521,* 1985) or in the RSV-LTR (from Rous sarcoma virus) (Gorman, CM., *Proc. Natl. Acad. ScL USA*

79:6777, 1982). Also useful are the promoter of the mouse metallothionein I gene (Hamer, D, *et al, J. MoI. Appl. Gen.* i:273-88, 1982; the TK promoter of Herpes virus (McKnight, S, *Cell 31:355-65,* 1982); the SV40 early promoter (Benoist, C , *et al, Nature* 290:304-10, 1981); and the yeast *gal4* gene promoter (Johnston, SA *et al, Proc. Natl. Acad. Sci. USA* 79:6971-5, 1982); Silver, PA, *et al, Proc. Natl. Acad. ScL (USA) 81:5951-5,* 1984)). Other illustrative descriptions of transcriptional factor association with promoter regions and the separate activation and DNA binding of transcription factors include: Keegan *et al, Nature 231:699,* 1986; Fields *et al, Nature 340:245,* 1989; Jones, *Cell 61:9,* 1990; Lewin, Cell 67:1161, 1990; Ptashne *et al, Nature 346:329,* 1990; Adams *et al, Cell* 72:306, 1993. The relevant disclosure of all of these above-listed references is hereby incorporated by reference.

The promoter region may further include an octamer region which may also function as a tissue specific enhancer, by interacting with certain proteins found in the specific tissue. The enhancer domain of the DNA construct of the present invention is one which is specific for the target cells to be transfected, or is highly activated by cellular factors of such target cells. Examples of vectors (plasmid or retrovirus) are disclosed in (Roy-Burman *et al,* U.S. Patent No. 5,1 12,767). For a general discussion of enhancers and their actions in transcription, see, Lewin, BM, *Genes IV,* Oxford University Press pp. 552-576, 1990 (or later edition). Particularly useful are retroviral enhancers *(e.g.,* viral LTR) that is preferably placed upstream from the promoter with which it interacts to stimulate gene expression. For use with retroviral vectors, the endogenous viral LTR may be rendered enhancer-less and substituted with other desired enhancer sequences which confer tissue specificity or other desirable properties such as transcriptional efficiency.

Nucleic acids of the invention can also be chemically synthesized using standard techniques, including solid-phase synthesis which, like peptide synthesis, has been fully automated with commercially available DNA synthesizers (Itakura U.S. Pats. No. 4,598,049, 4,401,796 and 4,373,071; Caruthers *et al.* U.S. Pat. No. 4,458,066.

PROTEINS AND POLYPEPTIDES

The terms "polypeptide," "protein," and "peptide" when referring to compositions of the invention are meant to include variants, analogues, and mimetics with structures and/or activity that substantially correspond to the polypeptide or peptide from which the variant, *etc.,* was derived.

The present invention includes an "isolated" fusion polypeptide comprising a targeting polypeptide linked to an antigenic polypeptide.

The term "chimeric" or "fusion" polypeptide or protein refers to a composition comprising at least one polypeptide or peptide sequence or domain that is chemically bound in a linear fashion with a second polypeptide or peptide domain. One embodiment of this invention is an isolated or recombinant

nucleic acid molecule encoding a fusion protein comprising at least two domains, wherein the first domain comprises an IPP and the second domain comprises an antigenic epitope, *e.g.,* an MHC class I-binding peptide epitope. The "fusion" can be an association generated by a peptide bond, a chemical linking, a charge interaction *(e.g.,* electrostatic attractions, such as salt bridges, H-bonding, *etc.)* or the like. If the polypeptides are recombinant, the "fusion protein" can be translated from a common mRNA. Alternatively, the compositions of the domains can be linked by any chemical or electrostatic means. The chimeric molecules of the invention (e.g., targeting polypeptide fusion proteins) can also include additional sequences, *e.g.,* linkers, epitope tags, enzyme cleavage recognition sequences, signal sequences, secretion signals, and the like. Alternatively, a peptide can be linked to a carrier simply to facilitate manipulation or identification/ location of the peptide.

Also included is a "functional derivative" of an IPP (or of its coding sequence) which refers to an amino acid substitution variant, a "fragment," or a "chemical derivative" of the protein, which terms are defined below. A functional derivative of an IPP retains measurable activity, preferably that is manifest as promoting immunogenicity of one or more antigenic epitopes fused thereto or co-administered therewith. "Functional derivatives" encompass "variants" and "fragments" regardless of whether the terms are used in the conjunctive or the alternative herein.

A functional homologue must possess the above biochemical and biological activity. In view of this functional characterization, use of homologous proteins including proteins not yet discovered, fall within the scope of the invention if these proteins have sequence similarity and the recited biochemical and biological activity.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes *(e.g.,* gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred method of alignment, Cys residues are aligned.

In a preferred embodiment, the length of a sequence being compared is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence. The amino acid residues (or nucleotides) at corresponding amino acid (or nucleotide) positions are then compared. When a position in the first sequence is occupied by the same amino acid residue (or nucleotide) as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the

sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* 45:444-453 (1970) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:1 1-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases, for example, to identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol.* 275:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a reference nucleic acid molecules. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to HVP22 protein molecules. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs *(e.g.,* XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

Thus, a homologue of a particular IPP as described herein is characterized as having (a) functional activity of the native IPP and (b) sequence similarity to a native IPP when determined as above, of at least about 20% (at the amino acid level), preferably at least about 40%, more preferably at least about 70%, even more preferably at least about 90%.

It is within the skill in the art to obtain and express such a protein using DNA probes based on the disclosed sequences.

Then, the chimeric DNA construct or fusion protein's biological activity can be tested readily using art-recognized methods such as those described herein in the Examples. A biological assay of the

stimulation of antigen-specific T cell reactivity will indicate whether the homologue has the requisite activity to qualify as a "functional" homologue.

A "variant" refers to a molecule substantially identical to either the full protein or to a fragment thereof in which one or more amino acid residues have been replaced (substitution variant) or which has one or several residues deleted (deletion variant) or added (addition variant). A "fragment" of the IPP refers to any subset of the molecule, that is, a shorter polypeptide of the full-length protein.

A preferred group of conservative variants are those in which at least one amino acid residue and preferably, only one, has been substituted by different residue. For a detailed description of protein chemistry and structure, see Schulz, GE *et al, Principles of Protein Structure,* Springer-Verlag, New York, 1978, and Creighton, T.E., *Proteins: Structure and Molecular Properties,* W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. The types of substitutions that may be made in the protein molecule may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz *et al. (supra)* and Figure 3-9 of Creighton *(supra).* Based on such an analysis, conservative substitutions are defined herein as exchanges within one of the following five groups:

1	Small aliphatic, nonpolar or slightly polar residues	Ala, Ser, Thr (Pro, Gly);
2	Polar, negatively charged residues and their amides	Asp, Asn, Glu, Gln;
3	Polar, positively charged residues	His, Arg, Lys;
4	Large aliphatic, nonpolar residues	Met, Leu, Ile, Val (Cys)
5	Large aromatic residues	Phe, Tyr, Trp.

The three amino acid residues in parentheses above have special roles in protein architecture. GIy is the only residue lacking a side chain and thus imparts flexibility to the chain. Pro, because of its unusual geometry, tightly constrains the chain. Cys can participate in disulfide bond formation, which is important in protein folding.

More substantial changes in biochemical, functional (or immunological) properties are made by selecting substitutions that are less conservative, such as between, rather than within, the above five groups. Such changes will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Examples of such substitutions are (i) substitution of GIy and/or Pro by another amino acid or deletion or insertion of GIy or Pro; (ii) substitution of a hydrophilic residue, *e.g.,* Ser or Thr, for (or by) a hydrophobic residue, *e.g.,* Leu, He, Phe, VaI or Ala; (iii) substitution of a Cys residue for (or by) any other residue; (iv) substitution of a residue having an electropositive side chain, *e.g.,* Lys, Arg or His, for (or by) a

residue having an electronegative charge, *e.g.,* GIu or Asp; or (v) substitution of a residue having a bulky side chain, *e.g.,* Phe, for (or by) a residue not having such a side chain, *e.g.,* GIy.

Most acceptable deletions, insertions and substitutions according to the present invention are those that do not produce radical changes in the characteristics of the wild-type or native IPP in terms of its intracellular processing, intercellular translocation, or other activity that is responsible for its ability to stimulate antigen specific T cell reactivity to an antigenic epitope or epitopes that are fused to the IPP. However, when it is difficult to predict the exact effect of the substitution, deletion or insertion in advance of doing so, one skilled in the art will appreciate that the effect can be evaluated by routine screening assays such as those described here, without requiring undue experimentation.

The term "chemically linked" refers to any chemical bonding of two moieties, *e.g.,* as in one embodiment of the invention, where a translocation polypeptide is chemically linked to an antigenic peptide. Such chemical linking includes the peptide bonds of a recombinantly or *in vivo* generated fusion protein.

THERAPEUTIC COMPOSITIONS AND THEIR ADMINISTRATION

A vaccine composition comprising the nucleic acid encoding the antigen or the antigen in a fusion polypeptide with an IPP, a particle comprising the nucleic acid or a cell expressing this nucleic acid, is administered to a mammalian subject, preferably a human together with an siNA, preferably an siRNA, that targets mRNA for a pro-apoptotic protein, preferably Bak and/or Bax. Another embodiment is a vaccine composition comprising DCs that are loaded with the antigen and transfected with the above siNA. The vaccine composition and siNA or the modified DCs are administered in a pharmaceutically acceptable carrier in a biologically effective or a therapeutically effective amount.

Certain preferred conditions are disclosed in the Examples. The composition may be given alone or in combination with another protein or peptide such as an immunostimulatory molecule. Treatment may include administration of an adjuvant, used in its broadest sense to include any nonspecific immune stimulating compound such as an interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether.

A therapeutically effective amount is a dosage that, when given for an effective period of time, achieves the desired immunological or clinical effect.

A therapeutically active amount of a nucleic acid encoding the fusion polypeptide may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the peptide to elicit a desired response in the individual. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. A

therapeutically effective amounts of the protein, in cell associated form may be stated in terms of the protein or cell equivalents.

Thus an effective amount of the vaccine and the siNA are between about 1 nanogram and about 1 gram per kilogram of body weight of the recipient, more preferably between about 0.1 μg/kg and about 10mg/kg, more preferably between about 1 μg/kg and about 1 mg/kg. Dosage forms suitable for internal administration preferably contain (for the latter dose range) from about 0.1 μg to 100 μg of active ingredient per unit. The active ingredient may vary from 0.5 to 95% by weight based on the total weight of the composition. Alternatively, an effective dose of DCs loaded with the antigen and expressing siRNA is between about 10^4 and 10^8 cells. Those skilled in the art of immunotherapy will be able to adjust these doses without undue experimentation.

The composition may be administered in a convenient manner, *e.g.,* injection by a convenient and effective route. Preferred routes for the DNA/ siRNA combination include intradermal "gene gun" delivery or intramuscular injection. The modified DCs are preferably administered by subcutaneous, intravenous or intramuscular routes. Other possible routes include oral administration, intrathecal, inhalation, transdermal application, or rectal administration. For the treatment of existing tumors which have not been completely resected or which have recurred, direct intratumoral injection is also intended.

Depending on the route of administration, the composition may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. Thus it may be necessary to coat the composition with, or co-administer the composition with, a material to prevent its inactivation. For example, an enzyme inhibitors of nucleases or proteases *(e.g.,* pancreatic trypsin inhibitor, diisopropylfluorophosphate and trasylol).or in an appropriate carrier such as liposomes (including water-in-oil-in-water emulsions as well as conventional liposomes (Strejan *et al, J. Neuroimmunol* 7:27, 1984).

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Preferred pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Pharmaceutical compositions suitable for injection include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. Isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride may be included in the pharmaceutical composition. In all cases, the composition

should be sterile and should be fluid. It should be stable under the conditions of manufacture and storage and must include preservatives that prevent contamination with microorganisms such as bacteria and fungi. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

Prevention of the action of microorganisms in the pharmaceutical composition can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like.

Compositions are preferably formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form refers to physically discrete units suited as unitary dosages for a mammalian subject; each unit contains a predetermined quantity of active material *(e.g.,* the nucleic acid vaccine) calculated to produce the desired therapeutic effect, in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of, and sensitivity of, individual subjects

For lung instillation, aerosolized solutions are used. In a sprayable aerosol preparations, the active protein may be in combination with a solid or liquid inert carrier material. This may also be packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant. The aerosol preparations can contain solvents, buffers, surfactants, and antioxidants in addition to the protein of the invention.

Other pharmaceutically acceptable carriers for the nucleic acid vaccine compositions according to the present invention are liposomes, pharmaceutical compositions in which the active protein is contained either dispersed or variously present in corpuscles consisting of aqueous concentric layers adherent to lipidic layers. The active protein is preferably present in the aqueous layer and in the lipidic layer, inside or outside, or, in any event, in the non-homogeneous system generally known as a liposomic suspension. The hydrophobic layer, or lipidic layer, generally, but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surface active substances such as dicetylphosphate, stearylamine or phosphatidic acid, and/or other

materials of a hydrophobic nature. Those skilled in the art will appreciate other suitable embodiments of the present liposomal formulations.

DELIVERY OF VACCINE NUCLEIC ACID TO CELLS AND ANIMALS

The following references set forth principles and current information in the field of basic, medical and veterinary virology and are incorporated by reference: *Fields Virology,* Fields, BN *et al,* eds., Lippincott Williams & Wilkins, NY, 1996; *Principles of Virology: Molecular Biology, Pathogenesis, and Control, Flint,* SJ. *et al,* eds., Amer Soc Microbiol, Washington DC, 1999; *Principles and Practice of Clinical Virology,* 4th Edition, Zuckerman. AJ. *et al,* eds, John Wiley & Sons, NY, 1999; *The Hepatitis C Viruses,* by Hagedorn, CH *et al,* eds., Springer Verlag, 1999; *Hepatitis B Virus: Molecular Mechanisms in Disease and Novel Strategies for Therapy,* Koshy, R. *et al,* eds, World Scientific Pub Co, 1998; *Veterinary Virology,* Murphy, F.A. *et al,* eds., Academic Press, NY, 1999; *Avian Viruses: Function and* CWroZJiitchie, B.W., Iowa State University Press, Ames , 2000; *Virus Taxonomy: Classification and Nomenclature of Viruses: Seventh Report of the International Committee on Taxonomy of Viruses,* by M. H. V. Van Regenmortel, MHV *et al,* eds., Academic Press; NY, 2000.

The Examples below describe certain preferred approaches to delivery of the vaccines and combinations of the present invention. A broader description of other approaches including viral and nonviral vectors and delivery mechanisms follow.

DNA delivery involves introduction of a "foreign" DNA into a cell *ex vivo* and ultimately, into a live animal or directly into the animal. Several general strategies for gene delivery (= delivery of nucleic acid vectors) for purposes that include "gene therapy" have been studied and reviewed extensively (Yang, N-S., *Crit. Rev. Biotechnol.* 72:335-356 (1992); Anderson, WF, *Science* 25(5:808-13, 1992; Miller, AS, *Nature* 357:455-60, 1992; Crystal, RG, *Amer. J. Med. 92(suppl* <5A):44-52S, 1992; Zwiebel, JA *et al, Ann NYAcad Sc. 618:394-404,* 1991; McLachlin, JR *et al, Prog. Nucl. Acid Res. Molec. Biol* 3δ:91-135, 1990; Kohn, DB *et al, Cancer Invest.* 7:179-92, 1989), which references are herein incorporated by reference in their entirety).

One approach comprises nucleic acid transfer into primary cells in culture followed by autologous transplantation of the *ex vivo* transformed cells into the host, either systemically or into a particular organ or tissue.

The term "systemic administration" refers to administration of a composition or agent such as a molecular vaccine as described herein, in a manner that results in the introduction of the composition into the subject's circulatory system or otherwise permits its spread throughout the body. "Regional" administration refers to administration into a specific, and somewhat more limited, anatomical space, such as intraperitoneal, intrathecal, subdural, or to a specific organ. The term "local administration"

refers to administration of a composition or drug into a limited, or circumscribed, anatomic space, such as intratumoral injection into a tumor mass, subcutaneous injections, intramuscular injections. One of skill in the art would understand that local administration or regional administration may also result in entry of a composition into the circulatory system.

For accomplishing the objectives of the present invention, nucleic acid therapy would be accomplished by direct transfer of a the functionally active DNA into mammalian somatic tissue or organ *in vivo*. DNA transfer can be achieved using a number of approaches described below. These systems can be tested for successful expression *in vitro* by use of a selectable marker *(e.g.,* G418 resistance) to select transfected clones expressing the DNA, followed by detection of the presence of the antigen-containing expression product (after treatment with the inducer in the case of an inducible system) using an antibody to the product in an appropriate immunoassay. Efficiency of the procedure, including DNA uptake, plasmid integration and stability of integrated plasmids, can be improved by linearizing the plasmid DNA using known methods, and co-transfection using high molecular weight mammalian DNA as a "carrier".

The DNA molecules encoding the fusion polypeptides of the present invention may be packaged into retrovirus vectors using packaging cell lines that produce replication-defective retroviruses, as is well-known in the art (see, for example, Cone, R.D. *et al, Proc. Natl. Acad. ScL USA* 81:6349-6353 (1984); Mann, R.F. *et at, Cell* 33:153-159 (1983); Miller, A.D. *et al., Molec. Cell. Biol.* 5:431-437 (1985),; Sorge, J., *et at, Molec. Cell. Biol.* 4:1730-1737 (1984); Hock, R.A. *et al, Nature* 320:257 (1986); Miller, A.D. *et al, Molec. Cell. Biol.* 6:2895-2902 (1986). Newer packaging cell lines which are efficient an safe for gene transfer have also been described (Bank *et al,* U.S. 5,278,056.

This approach can be utilized in a site specific manner to deliver the retroviral vector to the tissue or organ of choice. Thus, for example, a catheter delivery system can be used (Nabel, EG *et al, Science 244:1342* (1989)). Such methods, using either a retroviral vector or a liposome vector, are particularly useful to deliver the nucleic acid to be expressed to a blood vessel wall, or into the blood circulation of a tumor.

Other virus vectors may also be used, including recombinant adenoviruses (Horowitz, MS, In: *Virology,* Fields, BN *et al,* eds, Raven Press, NY, 1990, p. 1679; Berkner, KL, *Biotechniques* (5:616-29, 1988; Strauss, SE, In: *The Adenoviruses,* Ginsberg, HS, ed., Plenum Press, NY, 1984, chapter 11), herpes simplex virus (HSV) for neuron-specific delivery and persistence. Advantages of adenovirus vectors for human gene delivery include the fact that recombination is rare, no human malignancies are known to be associated with such viruses, the adenovirus genome is double stranded DNA which can be manipulated to accept foreign genes of up to 7.5 kb in size, and live adenovirus is a safe human vaccine

organisms. Adeno-associated virus is also useful for human therapy (Samulski, RJ *et al, EMBO J. 10:3941,* 1991) according to the present invention.

Another vector which can express the DNA molecule of the present invention, and is useful in the present therapeutic setting is vaccinia virus, which can be rendered non-replicating (U.S. Pats. 5,225,336; 5,204,243; 5,155,020; 4,769,330; Sutter, G *etal, Proc Natl Acad Sci USA* «9:10847-51, 1992; Fuerst, TR *et al, Proc. Natl. Acad. Sci. USA* 86:2549-53, 1992; Falkner FG *et al; Nucl. Acids Res 15:7192,* 1987; Chakrabarti, S *et al, MoI Cell Biol* 5:3403-9, 1985). Descriptions of recombinant vaccinia viruses and other viruses containing heterologous DNA and their uses in immunization and DNA therapy are reviewed in: Moss, B, *Curr Opin Genet Dev* 3:86-90, 1993; Moss, B, *Biotechnol.* 20:345-62, 1992); Moss, B, *Curr Top Microbiol Immunol* 758:25-38, 1992; Moss, B, *Science* 252:1662-7, 1991; Piccini, *A et al.,Adv. Virus Res 34:43-64,* 1988; Moss, B *et al, Gene Amplif Anal* 3:201-13, 1983).

In addition to naked DNA or RNA, or viral vectors, engineered bacteria may be used as vectors. A number of bacterial strains including *Salmonella,* BCG and *Listeria monocytogenes(LM)* (Hoiseth *et al, Nature 291:238-239,* 1981; Poirier, TP *et al, J. Exp. Med. 168:25-32,* 1988); Sadoff, JC *et al, Science* 240:336-8, 1988; Stover, CK *et al, Nature* 357:456-60, 1991; Aldovini, A *et al, Nature* 357:479-82, 1991; Schafer, R, *et al, J Immunol 149:53-9* (1992); Ikonomidis, G *et al, J Exp Med 180* :2209-18, 1994). These organisms display two promising characteristics for use as vaccine vectors: (1) enteric routes of infection, providing the possibility of oral vaccine delivery; and (2) infection of monocytes/macrophages thereby targeting antigens to professional APCs.

In addition to virus-mediated gene transfer *in vivo,* physical means well-known in the art can be used for direct transfer of DNA, including administration of plasmid DNA (Wolff *et al,* 1990, *supra)* and particle-bombardment mediated gene transfer (Yang, N-S, *et al, Proc Natl Acad Sci USA* 87:9568, 1990; Williams, RS *et al, Proc Natl Acad Sci USA* 88:2726, 1991; Zelenin, AV *et al, FEBS Lett* 280:94 , 1991; Zelenin, AV *et al, FEBS Lett 244:65,* 1989); Johnston, SA *et al, In Vitro Cell Dev Biol* 27:11, 1991). Furthermore, electroporation, a well-known means to transfer genes into cell *in vitro,* can be used to transfer DNA molecules according to the present invention to tissues *in vivo* (Titomirov, AV *et al, Biochim BiophysActa* 7088:131, 1991).

"Carrier mediated gene transfer" has also been described (Wu, CH *et al, J Biol Chem* 2(54:16985, 1989; Wu, GY *et al, J Biol Chem 263:14621,* 1988; Soriano, P *et al, Proc Nat. Acad Sci USA* 80:7128, 1983; Wang, C-Y *et al, Pro. Natl Acad Sci USA* 84:7851, 1982; Wilson, JM *et al, JBiol Chem 267:963,* 1992). Preferred carriers are targeted liposomes (Nicolau, C *et al, Proc Natl Acad Sci USA* 80:1068, 1983; Soriano *et al, supra)* such as immunoliposomes, which can incorporate acylated mAbs into the lipid bilayer (Wang *et al, supra).* Polycations such as asialoglycoprotein/polylysine (Wu

et al, 1989, *supra)* may be used, where the conjugate includes a target tissue-recognizing molecule *{e.g.,* asialo-orosomucoid for liver) and a DNA binding compound to bind to the DNA to be transfected without causing damage, such as polylysine. This conjugate is then complexed with plasmid DNA of the present invention.

Plasmid DNA used for transfection or microinjection may be prepared using methods well-known in the art, for example using the Quiagen procedure (Quiagen), followed by DNA purification using known methods, such as the methods exemplified herein.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE 1

Materials and Methods

Plasmid DNA Constructs and DNA Preparation: The production of the following vectors have been described: pcDNA3-E7, pcDNA3-Sig/E7/LAMP-l, pcDNA3-E7/HSP70, pcDNA3-CRT/E7, pDNA3-E7/GFP and pcDNA3-OVA (Kim TW *et al, J Clin Invest 112:* 109-1 17, 2003; Cheng WF *et al, J Clin Invest 108:* 669-678, 2001; Hung CF *et al, Cancer Res 61:* 3698-3703., 2001; Chen CH *et al, Cancer Res 60:* 1035-1042, 2000; see also US Pat. 6,734,173 and published patent applications WO05/081716, WO05/047501, WO03/085085, WO02/12281C2, WO02/074920, WO02/061113, WO02/09645, and WO01/29233. The plasmid containing a sequence encoding influenza hemagglutinin (HA), pcDNA3-HA, was provided by Dr. Drew Pardoll Johns Hopkins School of Medicine. The accuracy of these constructs was confirmed by DNA sequencing. DNA was amplified in *E. coli* DH5α and purified as described in Chen, CH et al., *supra).*

Preparation (synthesis) of siRNAs and Transfection: siRNAs were synthesized using 2'-0-ACE-RNA phosphoramides (Dharmacon, Lafayette, Colorado). The sense and anti-sense strands of siRNA were :

Gene targeted	siRNA Sequence	SEQ ID NO:
Bak, beginning at nt 310,	5'- UGCCUACGAACUCUUCACCdTdT-3' (sense)	1
	5'-GGUGAAGAGUUCGUAGGCAdTdT-3' (antisense)	2
Bax, beginning at nt 217,	5'-UAUGGAGCUGCAGAGGAUGdTdT-3' (sense)	5
	5'-CAUCCUCUGCAGCUCCAUAdTdT-3' (antisense)	6
Non-specific ctrl siRNA	5'-NNATTGTATGCGATCGCAGAC-3'	45

RNAs were deprotected and annealed according to the manufacturer's instruction. Non-specific control siRNA was acquired from Dharmacon.

Dendritic cells - either DC-I cells or bone marrow-derived DCs (BM-DCs) incubated for 6 days were transfected with Bak and Bax siRNA or control siRNA using Oligofectamine (Invitrogen, Carlsbad, CA). 24 to 48 hours later, the transfected cells were used.

Cells: The HPV-16 E7-expressing murine tumor model, TC-I, has been described previously. In brief, HPV-16 E6, E7, and the *ras* oncogene were used to transform primary C57BL/6 mouse lung epithelial cells to generate TC-I.

DC-I cells were generated from the dendritic cell line provided by Dr. Kenneth Rock, University of Massachusetts. With continued passage, subclones of DCs (DC-I) have been generated that can be easily transfected (Kim *et at,* 2004, *supra).*

Cells were maintained in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 2mM glutamine, ImM sodium pyruvate, lOOμM non-essential amino acids, 2OmM HEPES, 50μM β-mercaptoethanol, 100 IU/ml penicillin, lOOμg/ml streptomycin and 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA).

An H-2D^b-restricted HPV-16 E7-specific T cell line has also been described previously (Wang, TL *et at, Gene Ther* 7:726-733, 2000). These cell lines were stimulated weekly with irradiated TC-I cells and 20U/ml murine rIL-2 weekly.

Generation of Bone Marrow-derived DCs (BM-DCs): BM-DCs were generated from bone marrow (BM) progenitor cells generally as described by Inaba *et at {J Exp Med 176:* 1693-1702, 1992) with a modification. Briefly, BM cells were flushed from femurs and tibias of 5-8-wk old C57BL/6 mice. Cells were washed twice with RPMI-1640 after lysis of red blood cells, and were resuspended at a density of 10^6/ml in RPMI-1640 medium supplemented as above, although with 5% fetal bovine serum, and further with 20ng/ml recombinant murine GM-CSF (PeproTech, Rock Hill, NJ). The cells were cultured in 24-well plates (lml/well) at 37°C in 5% humidified CO_2. Wells were replenished on days 2 and 4 with fresh medium supplemented GM-CSF as above. Cells were harvested after 6 days and subjected to transfection with siRNA.

Western Blot Analysis: 2xlO^5 DC-I cells were transfected with 300 pmol of the Bak+Bax siRNA or control siRNA in a final volume of 2ml using Oligofectamine® (Invitrogen, Carlsbad, CA) according to vendor's instructions. Fluorescein-labeled siRNA was used to assess the transfection efficiency of DC-I cells by flow cytometric analysis. Virtually 100% of DC-I cells were successfully transfected with siRNA. The expression of Bak and Bax pro-apoptotic proteins in DC-I cells transfected with Bak and/or Bax siRNA was characterized by Western blot analysis using 50μg of cell lysate from transfected DC-I

cells and anti-Bak and/or anti-Bax mouse mAb (Cell Signaling Technology, Inc., Beverly, MA) using a protocol similar to that described previously (Hung *et al,* 2001, *supra).*

Measurement of Apoptotic Cells; As described, $2x10^5$ DC-I cells were transfected with Bak+Bax siRNA or control siRNA. Two days after transfection, the cells were pulsed with 10 μg/ml E7 peptide (RAHYNIVTF; SEQ ID NO:46) or HA peptide (IYSTVASSL; SEQ ID NO:47) for 2 hours and subsequently incubated with an E7-specific $CD8^+$ T cell line (Wang TL *et al,* 2000, *supra)* at different E:T ratios (5, 1, 0.5 and 0.1) for 4 or 20 hrs. Apoptotic DC-I cells were detected using PE-conjugated rabbit anti-active caspase-3 mAb (BD Pharmingen San Diego CA) according to the vendor's protocol. Briefly, cells were harvested and stained with FITC-conjugated anti-CD8 antibody as described previously. The cells were subsequently fixed and permeabilized using the Cytofix/Cytoperm™ Kit (BD Pharmingen) for 20 minutes at room temperature, and stained with PE-conjugated rabbit-anti-active caspase-3 monoclonal antibody using 20 μl per 10^6 cells for 60 minutes at room temperature. Following incubation with the antibodies, the cells were washed, resuspended and analyzed by flow cytometric analysis. Analysis was performed on a Becton-Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA). $CD8^{nes}$ cells were gated and active caspase-3-positive DC-I cells were analyzed to determine the percentage of apoptotic DC-I cells.

Mice: C57BL/6 mice (6- to 8-week-old) were purchased from the National Cancer Institute (Frederick, MD) and maintained under specific pathogen-free conditions in the oncology animal facility of the Johns Hopkins Medical Institutions (Baltimore, Maryland). All procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

DNA/siRNA Vaccination: Gene gun particle-mediated DNA/siRNA vaccination was performed using a helium-driven gene gun (Bio-Rad, Hercules, CA) according to the protocol for RNA vaccination provided by the manufacturer, with a slight modification. Briefly, DNA/siRNA -coated gold particles were prepared by combining 25 mg of 1.6 μm gold microcarriers (Bio-Rad), 50 μg of plasmid DNA (50μl), 5μg of siRNA (50μl), and 10 μl of 3M sodium acetate. Isopropyl alcohol (200μl) was added to the mixture drop-wise while mixing by vortex. The mixture was allowed to precipitate at room temperature for 10 min. The suspension of microcarrier/DNA/siRNA was centrifuged 10,000 rpm for 30 s and washed 3 times in fresh absolute ethanol before resuspending in 3 ml of polyvinylpyrrolidone (0.1 mg/ml; Bio-Rad) in absolute ethanol. The solution was then loaded into 2.5 ft. of Gold-Coated™ tube (Bio Rad) and allowed to settle for 10 min. The ethanol was gently removed, and the microcarrier/DNA/siRNA suspension was evenly attached to the inside surface of the tube by rotation. The tube was then dried using flowing nitrogen gas at a rate of 0.4 liters/min. The dried tube coated with microcarrier/ DNA/siRNA was then cut into 0.5 inch cartridges and stored in a capped dry bottle at 4^0C.

The DNA/siRNA-coated gold particles (1 μg of DNA and 0.1 μg of siRNA /bullet) were delivered to the shaved abdomens of mice using a helium-driven gene gun *(supra)* with a discharge pressure of 400 psi. Mice were immunized with 2 μg of the desired pcDNA3 plasmid, including those encoding E7, Sig/E7/LAMP-1, E7/HSP70, CRT/E7, HA, or OVA, mixed with 0.2 μg of Bak+Bax siRNA or control siRNA. The mice were boosted with the same dose 1 wk later.

To determine the effect of Bak+Bax siRNA and/or control siRNA administered during priming and/or boosting phases, mice were primed with 2 μg of pcDNA3-Sig/E7/LAMP-l co-administrated with 0.2 μg of Bak+Bax siRNA or with control siRNA. Mice were then boosted with 2 μg of pcDNA3-Sig/E7/LAMP-1 co-administrated with 0.2 μg of Bak+Bax siRNA or control siRNA.

DC Immunization: DC-I cells or BM-DCs were transfected with the Bak+Bax siRNA or control siRNA as above. Two days later, DC-I cells or BM-DCs transfected with Bak/Bax siRNA or with control siRNA were incubated with E7 aa49-57 peptide (RAHYNIVTF; SEQ ID NO:46) (lOμg/ml) at 37°C for 2 hours. The cells were then washed with RPMI-1640/10% FCS and HBBS, and resuspended in HBBS at the final concentration of 5 X 10^6/ml (DC-I cells) or 2xl0 ^{6}/ml (BM-DCs). DC-I cells or BM-DCs were injected s.c. into footpads of mice (lOOμl/mouse). One week later, the mice were boosted once with the same dose and immunization regimen.

Intracellular Cytokine Staining (ICCS) and Flow cytometric analysis; Spleen cells were harvested from mice one week after the last vaccination. Prior to ICCS, 4xlO^6 (or 3.5 x 10^5) pooled spleen cells from each treatment group were incubated overnight or for about 16 hours with

(a) 1 μg/ml of E7 peptide (RAHYNIVTF; SEQ ID NO:46), HA (IYSTVASSL; SEQ ID NO:47) (underscored in SEQ ID NO:23), or OVA peptide (SIINFEKL; SEQ ID NO:48), each of which includes an MHC class I epitope, to detect antigen-specific $CD8^+$ T cell precursors; or

(b) 1 μg/ml of E7 peptide (aa 30-67) containing an MHC class II epitope - DSSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDSTLRL (SEQ ID NO:49) - for detection of antigen-specific CD4+ T cell precursors. Intracellular IL-4 and IFN-γ staining and flow cytometric analysis were performed as described previously.

In studies of DC-I or BM-DC vaccination, 3.5 X 10^5 pooled spleen cells from each group (see above) were used. GolgiPlug (BD Pharmingen) was added to the culture, and incubated at 37°C overnight. Cells were then washed once with FACScan® buffer and stained with phycoerythrin-conjugated monoclonal rat antimouse CD8a antibody (clone 53.6.7). Cells were subjected to ICCS using the Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen). Intracellular IFN-γ was stained with FITC-conjugated rat antimouse IFN-γ. Analysis of surface markers of untransfected or siRNA-transfected DCs was performed on FACS Calibur and analyzed using CellQuest

software (BD Bioscience, San Jose, CA). FITC-conjugated mouse mAbs specific for the surface markers CDl Ic, CD40, CD86, 1-A^b, or H-$2K^b/D^b$ (BD Pharmingen) were used.

***In Vivo* Tumor Protection and Tumor Treatment Experiments:** For tumor protection studies, C57BL/6 mice (5/group) were challenged s.c, with $5xlO^4$ TC-I tumor cells/mouse in the right leg one week after the last vaccination. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week. To evaluate lymphocytes subsets responsible for antitumor effects, *in vivo* antibody depletion studies were performed using standard methods *(e.g.,* Lin KY *et al, Cane Res 56:21-*6., 1996).

For tumor therapy studies, mice were challenged with 1 or $5xlO^4$ TC-I tumor cells/mouse Lv., in the tail vein to simulate hematogenous spread of tumors (Ji *et al, supra).* Mice were treated three days after tumor challenge with (a) DNA vaccine mixed with siRNA, boosted once after 1 wk and sacrificed on day 42 after the last vaccination or (b) 5 x 10^5 E7 peptide-pulsed siRNA-transfected DC-I, boosted once after 1 wk and sacrificed on day 28 after the last immunization. The mean number of pulmonary nodules in each mouse was evaluated by experimenters blinded to sample identity. *In vivo* tumor protection, antibody depletion, and tumor therapy experiments were performed at least two times to generate reproducible data.

Preparation of CDllc $^+$ Cells from Inguinal Lymph Nodes of Vaccinated Mice: C57BL/6 mice (5/group) were first primed with pcDNA3-Sig/E7/LAMPl or control pcDNA3 DNA via gene gun at a dose of 2 μg/mouse. Seven days later, mice received 16 inoculations of non-overlapping gene gun intradermal administration on their abdomens. Gold particles used for each inoculation were coated with 1 μg of pcDNA3-E7/GFP DNA mixed with 0.1 μg of Bak+Bax siRNA or control siRNA. pcDNA3 mixed with Bak+Bax siRNA was used as a negative control.

Inguinal lymph nodes (LN) draining the inoculation site were harvested from vaccinated mice 2 or 5 days after vaccination. CDl Ic^+ cells were enriched from a single cell suspension of isolated LN cells using CDl Ic (N418) microbeads (Miltenyi Biotec, Auburn, CA). Enriched CDl Ic^+ cells were analyzed by forward and side scatter and gated around a population of cells with size and granular characteristics of DCs. GFP^+ cells were analyzed by flow cytometry using a protocol described previously (Lappin MB *et al, Immunology 98:* 181-8, 1999). Results are expressed as percent of GFP^+ CDl Ic^+ cells among gated monocytes. The percent of GFP^+ cells among the gated CDl Ic^+ cells was analyzed by flow cytometry.

In vivo antibody depletion studies were performed using conventional methods as noted above. Depletion was initiated 5 days after priming and terminated at time of LN harvest.

Adoptive transfer of T cells and rapid DC elimination assay: To create two distinctly labeled populations of BM-DCs, different concentrations of the dye carboxyfluorescein (CFSE) were used to label cells. E7-peptide-loaded BM-DCs transfected with either Bax/Bax siRNA or control siRNA were prepared using methods described above. The E7-peptide loaded BM-DCs transfected with control siRNA were labeled with 5μM CFSE ("high-CFSE") , whereas Bak/Bax siRNA-transfected DCs were labeled with 10-fold lower concentration, 0.5μM CFSE ("low-CFSE"). A 1:1 mixture of $2.5xlO^5$ low CFSE-labeled E7-peptide loaded BM-DCs and $2.5xlO^5$ high CFSE-labeled E7-peptide loaded BM-DCs was administered i.v. to C57BL/6 mice three days after adoptive transfer i.v. of 10^6 E7-specific T cells into the mice. Sixteen hours later, single cell suspensions from the lung and spleen were prepared and analyzed for CFSE content by flow cytometry.

Statistical Analysis: All results expressed as means ± standard errors (SE) are representative of at least two experiments. Results of ICCS with flow cytometric analysis and tumor treatment experiments were evaluated by analysis of variance (ANOVA). Comparisons between individual data points were made using Student's t-test. In tumor protection experiments, the principal outcome of interest was time to tumor development. The event time distributions for different mice were compared using the Kaplan and Meier method and the log-rank statistic. All p values <0.05 were considered significant.

EXAMPLE 2

Transfection with Bak and/or Bax siRNA Leads to Downregulation of Bak and Bax, and Resistance to Apoptotic Cell Death

To determine if the expression of Bak and/or Bax was downregulated in a DC cell line (DC-I) transfected with Bak and/or Bax siRNA, western blot analysis was performed using cell lysate from DC-1 cells, transfected with the various siRNAs. As shown in **Figure 1,** the expression of Bak and/or Bax proteins was undetectable in DC-I cells transfected with Bak and/or Bax siRNA. In contrast, expression of Bak and Bax proteins was detected in DC-I cells after transfection with control siRNA, the levels of expression being similar to the levels in nontransfected DC-I cells. The expression of β-actin protein was consistent among all DC-I cell groups. The kinetics of inhibition of Bak and Bax protein expression by DC-I cells transfected with Bak+Bax siRNA were examined. As shown in **Figure** 2, significant downregulation of Bax and Bak expression was observed 1 day after transfection. No Bak or Bax expression was detectable at days 3, 5, and 7 and some expression was detected at by day 9 (below-normal levels). Expression returned to normal levels by day 11 after transfection.

To determine if DC-I cells transfected with Bak and/or Bax siRNA could resist CTL-induced apoptosis, E7 peptide-loaded, siRNA-transfected DC-I cells were incubated with an E7-specific $CD8^+$ T cell line and the percentages of apoptotic cells was measured. As shown in **Figure 3A and 3B,** 80-90%

of E7 peptide-loaded DC-I cells transfected with control siRNA were apoptotic by 20 hrs. In comparison, fewer DC-I cells transfected with Bak+Bax siRNA were apoptotic, particularly at low E:T ratios (T cells to DC-I cells).

These results show that transfection of DC-I cells with Bak and/or Bax siRNA downregulates Bak and Bax protein expression, resulting in resistance to the apoptotic effects of activated, antigen-specific $CD8^+$ T cells on the DCs.

EXAMPLE 3

Coadministration of Bax+Bak siRNA with Antigen-specific DNA Vaccines Significantly Enhances Numbers of Antigen-Specific $CD8^+$ T cell Precursors in Vaccinated Mice

To determine if the anti-apoptotic action of Bak+Bax siRNA observed in DCs *in vitro* can be manifest *in vivo,* Bak+Bax siRNA was coadministered with pcDNA3-E7 intradermally via gene gun. As shown in **Figures 4 and 5,** coadministration of pcDNA3-E7 with Bak and/or Bax siRNA significantly enhanced the E7-specific $CD8^+$ T cell response (by at least 10-fold) in vaccinated mice, compared to coadministration of pcDNA3-E7 with control siRNA.

To determine if this result was obtained using other antigens, pcDNA3-HA and pcDNA3-OVA plasmids were coadministered with Bak+Bax siRNA. As shown in **Figures 6- 7,** the coadministration of pcDNA3-HA or pcDNA3-OVA with Bak+Bax siRNA significantly enhanced the HA- and OVA-specific $CD8^+$ T cell response in vaccinated mice, compared to coadministration of the antigen vectors with control siRNA. Thus Bak and/or Bax siRNA significantly enhance antigen-specific $CD8^+$ T cell-mediated immune responses when coadministered with antigen-encoding DNA vaccines.

EXAMPLE 4

Co-administration of Bak+Bax siRNA with an E7-specific DNA Vaccine Significantly Enhances Antitumor Effects Against an E7-expressing Target Tumor Cell Line

To determine if the observed enhancement of E7-specific T cell-mediated immunity described above can manifest itself in E7-specific antitumor effects, an *in vivo* tumor protection experiment was performed using E7-expressing TC-I tumor cells. As shown in **Figure** 8, all mice receiving E7 DNA mixed with Bak+Bax siRNA remained tumor-free for 35 days after TC-I challenge. In contrast, all of the mice receiving E7 DNA with control siRNA or pcDNA3 (negative control for antigen) combined with Bak+Bax siRNA developed tumors by day 10.

An *in vivo* antibody depletion experiment was conducted to determine which subsets of lymphocytes were responsible for the anti-tumor effects. As shown in **Figure** 9, 100% of mice depleted of $CD8^+$ T cells grew tumors within 10 days after TC-I challenge. In contrast, 100% of the mice

depleted of $CD4^+$ T cells or NK cells remained tumor-free 35 days after TC-I challenge (as with the "non-depleted mice discussed above). It was concluded that $CD8^+$ T cells are needed for the antitumor effects induced by the combination of a DNA vaccine and Bak+Bax siRNA.

An *in vivo* tumor therapy experiment was performed using a model of hematogenous spread of tumors to the lungs (Ji *et al, supra).* As shown in **Figure** 10, mice immunized with E7 DNA combined with Bak+Bax siRNA exhibited the fewest pulmonary tumor nodules ($p<0.005$) compared to mice vaccinated with E7 DNA combined with control siRNA, or pcDNA3 (no antigen) combined with Bak+Bax siRNA. Taken together, these results indicate that vaccination with the combination of E7 DNA with Bak+Bax siRNA leads to potent protective and therapeutic effects against E7-expressing TC-1 tumor cells.

EXAMPLE 5

Combined Application of Anti-Apoptotic Bak+Bax SiRNA and An Intracellular Targeting Strategy Enhances Antigen-Specific T Cell-Mediated Immune Responses

To assess the effect of coadministration of Bak+Bax siRNA with DNA encoding E7 linked to an DNA encoding an PP such as an intracellular targeting molecule, mice were vaccinated with either Sig/E7/LAMP-1 DNA, HSP70/E7 DNA, or CRT/E7 DNA each combined with either (i) Bak+Bax siRNA or (ii) control siRNA. As shown in **Figures** 11-12, coadministration of Bak+Bax siRNA with pcDNA3 encoding Sig/E7/LAMP-1, HSP70/E7, or CRT/E7 resulted in increased numbers of DFN-γ-expressing E7-specific $CD8^+$ T cell precursors compared to coadministration of each of these three constructs with control siRNA. Among these intracellular targeting strategies, mice vaccinated with pcDNA3-Sig/E7/LAMP-1 combined with Bak+Bax siRNA displayed the greatest increase in the number E7-specific $CD8^+$ T cell precursors (about a 19-fold). Thus, administration of Bak+Bax siRNA can be combined with any of the intracellular targeting strategies (using any IPP that itself can potentiate responses over those of DNA encoding antigen alone) to further enhance the potency of a DNA vaccine. Of the Antigen/IPP fusions tested, immunity was enhanced the most when pcDNA3-Sig/E7/LAMP-1 was combined with Bak+Bax siRNA.

The ability of the Sig/E7/LAMP-1 targeting strategy to enhance antigen presentation to $CD4^+$ T lymphocytes is achieved through targeting of expressed antigen to endosomal/lysosomal compartments, important loci for the MHC class U antigen presentation pathway (Wu TC *et al., Proc Natl Acad Sci USA 92:* 11671-5, 1995). As shown in **Figures** 13-14, vaccination with pcDNA3-Sig/E7/LAMP-1 combined with Bak+Bax siRNA generated significantly more E7-specific $CD4^+$ ThI cells and similar numbers of E7-specific $CD4^+$ Th2 cells when compared to vaccination with the same immunogen plus

control siRNA. These results show that coadministration of Sig/E7/LAMP-1 DNA with Bak+Bax siRNA elicits an immune response mediated predominantly by E7-specific $CD4^+$ ThI cells.

EXAMPLE 6

Co-Administration with Anti-Apoptotic Bax+Bak siRNA Improves Survival of DNA-Transfected DCs in Inguinal Lymph Nodes of Mice Vaccinated with E7/GFP DNA

Mice were primed with pcDNA3-Sig/E7/LAMP-l to generate sufficient E7-specific $CD8^+$ T cells for testing of the anti-apoptotic ability of Bak+Bax siRNA in E7/GFP-expressing DCs. pcDNA3 (plasmid only) was the negative control. One week later, mice were treated via gene gun with pcDNA3-E7/GFP DNA plus either Bax+Bak siRNA or control siRNA. As shown in **Figures 15-16,** two days after vaccination. Control groups primed with pcDNA3 showed no significant difference in the percentages of GFP^+ CDl Ic^+ DCs between mice that received Bak+Bax siRNA and in that received siRNA. In comparison, in mice primed with the DNA immunogen pcDNA3-Sig/E7/LAMP-l, a significant decrease was observed in the percentage of GFP^+ CDl Ic^+ DCs detected in mice receiving control siRNA vs. the percentage of GFP^+ CDl Ic^+ DCs in mice administered Bak+Bax siRNA. Five days after vaccination with pcDNA3-E7/GFP, a similar, albeit weaker trend was observed in mice primed with Sig/E7/LAMP~ 1.

Assays for apoptotic GFP^+ CDl Ic^+ DCs were performed by staining cells for activated caspase-3 followed by flow cytometry. More than 90% of GFP^+CDl Ic^+ DCs were caspase-3 negative, indicating that these cells were not apoptotic (not show). Thus, these results show that co-administration of anti-apoptotic Bak+Bax siRNA with the DNA immunogen E7/GFP protects DNA-transfected DCs from being killed by E7-specific $CD8^+$ T cells generated as a result of antigen-specific priming (by pcDNA3-Sig/E7/LAMP-1).

An antibody depletion experiment confirmed that $CD8^+$ T cells were responsible for the induction of apoptosis in GFP^+ CDl Ic^+ DCs. As shown in **Figure** 17, the percentages of GFP^+ CDl I^+ DCs in the inguinal LNs of mice depleted of $CD8^+$ T cells were similar in mice administered Bak+Bax siRNA compared to mice administered control siRNA. In comparison, percentages of GFP^+ CDl I^+ cells in the inguinal LNs of mice depleted of $CD4^+$ T cells, NK cells, or control mice (no depletion) were significantly lower in mice receiving control siRNA compared to mice receiving Bak+Bax siRNA ($p<0.005$). Thus, $CD8^+$ T cells are responsible for the induction of apoptosis in antigen-expressing DCs in the draining LNs of vaccinated mice.

EXAMPLE 7

Co-Administration of Bak+Bax siRNA with DNA Vaccines During Boosting Elicits a Stronger Antigen-Specific CD8+ T Cell Response than Co-Administration During Priming

The results shown in **Figures** 15-18, indicate that the anti-apoptotic siRNA strategy is most critical for prolonging DC life when a pre-existing active antigen-specific $CD8^+$ T cell population is present; this occurs in the boosting phase of the DNA vaccination protocols used here.

To determine whether prolonging the life of antigen-presenting DCs has more of an impact during the priming or the boosting phases of the vaccination process, pcDNA3-Sig/E7/LAMP-1 was co-administered with Bak+Bax siRNA or control siRNA during the priming or during the boosting phases. As shown in **Figures** 19-20, mice administered Bak+Bax siRNA during the priming and boosting phases generated the greatest number of E7-specific $CD8^+$ T cell precursors when compared to the other vaccination groups. Administration of Bak+Bax siRNA during the boosting phase resulted in a markedly higher number of E7-specific $CD8^+$ T cells than administration during the priming phase ($p=0.002$). These results show that prolonging the life of antigen-expressing DCs via administration of siRNA during the boosting phase has a greater impact on the (clonal) expansion of antigen-specific T cells.

EXAMPLE 8

Discussion of Examples 2-7

In vivo delivery of siRNA to target cells represents a significant challenge. Considerable endeavors have been devoted to efficient delivery of siRNA to specific cell types or organs *in vivo* (Song E *et al, Nat Med* 9:347-51, 2003). So far, these endeavors have met with only limited success (for a review, see Wall NR *et al, Lancet 362:1401-3,* 2003). The present inventors have shown that intradermal delivery to APCs via gene gun is an effective system for delivery of siRNA into professional antigen-presenting cells, allowing evaluation of siRNA-based strategies to modify DCs. Thus, the present work is the first to use intradermal delivery of siRNA to DCs and permits investigation of the properties of antigen-expressing DCs *in vivo.*

The encouraging results reported above indicate that modifying the function of DCs *in vivo* using siRNA technology targeting other key pro-apoptotic proteins, such as caspases 3, 6, 7, 8, or 9, should also enhance DNA vaccine potency. Furthermore, according to this invention, a combination of more than one type of siRNA targeting multiple pro-apoptotic proteins within the extrinsic and intrinsic apoptotic pathways is useful to induce even greater resistance to apoptotic stress in transfected DC-I cells. This should result in greater numbers of viable, functional antigen-expressing DCs in the LNs draining a site of immunization in effectively primed mice. Other cell surface molecules such as PD-Ll and PD-L2 (Khoury SJ *et al, Immunity* 20:529-38, 2004; Carreno BM *et al.,Annu Rev Immunol 20:29-*53, 2002) and/or cytokines, such as IL4 and IL-10 (Li-Weber M *et al, Nat Rev Immunol* 3:534-43, 2003;

Moore KW *et al.,Annu Rev Immunol 19* 683-765, 2001) expressed by DCs cells suppress T cell responses. Expression of these molecules can be silenced by the siRNA technology to enhance antigen specific immune responses and the resultant antitumor effects.

Disclosed above is a significant increase in the number of GFP-positive DCs in the draining LNs of vaccinated mice after coadministration of pcDNA3-E7/GFP with Bak+Bax siRNA, compared to coadministration of pcDNA3-E7/GFP and control siRNA. This increase is likely due to enhanced DC survival mediated by Bak+Bax siRNA, rather than an influence on migration of DCs cells due to some nonspecific siRNA effect. This is so because coadministration of pcDNA3-E7/GEP with control siRNA did not produce similar effects. Previous observations by the present inventors and colleagues using DNA-encoding anti-apoptotic proteins (Kim TW *et al, J Clin Invest,* 2003, *supra)* support such a notion. DNA vaccines encoding antigen were coadministered with DNA encoding BCL-xL to prolong the lives of transfected DCs. While co-administration to mice of DNA encoding antigen with DNA encoding BCL-xL led yielded increased number of antigen-expressing DCs in the draining LNs, coadministration of the same immunogen with DNA encoding *mutant* BCL-xL with minimal mutations in a region critical to anti-apoptotic function, failed to lead to such an increase. Thus, the increase in GFP-positive DCs in the draining LNs after co-administration of Bak+Bax siRNA discussed above can be ascribed to changes in survival of DCs.

The increased number of antigen-expressing DCs in the LNs following the coadministration of Bak+Bax siRNA can contribute to increased numbers of E7-specific $CD8^+$ T cells through multiple mechanisms. Not only do antigen-expressing DCs provide signals to trigger proliferation and expansion of antigen-specific T cells, but they also can provide necessary signals that reduce T cell apoptotic death. Normally, DC death leads to decreasing interaction between APCs and lymphocytes, causing T cells to downregulate anti-apoptotic molecules and potentially upregulate pro-apoptotic molecules (Opferman *et al, supra).* This process would naturally lead to a decline in number of activated antigen-specific $CD8^+$ T cells. The continued survival of antigen-expressing DCs thanks to siRNA-mediated silencing of pro-apoptotic molecules would provide the necessary signals to prevent this decline. Other explanations for enhanced T cell responses include qualitative changes in antigen-expressing DCs as a result of vaccination together with Bak+Bax siRNA administration. The present inventors have observed that antigen-expressing DCs transfected with Bak+Bax siRNA could activate antigen-specific $CD8^+$ T cells more efficiently than DCs transfected with control siRNA (not shown). Thus, the anti-apoptotic function mediated by Bak+Bax siRNA may modify the quantity and quality of DCs, thereby leading to enhanced T cell activation.

The present results show that prolonging the life of antigen-expressing DCs during the boosting phase is important for clonal expansion of antigen-specific T cells. Killing of antigen-expressing DCs is

a natural process that regulates clonal expansion of antigen-specific $CD8^+$ T cells. Pre-existing antigen-specific $CD8^+$ T cells in draining LNs can lyse antigen-expressing DCs, limiting clonal expansion (Ritchie DS *et al, J Immunol Meth 246:109-17,* 2000; Hermans IF *et al, J Immunol* 764:3095-3101, 2000). Such $CD8^+$ T cell-mediated lysis of DCs is more significant during the boosting phase of vaccination than during the priming phase, due to the increased number of antigen-specific $CD8^+$ T cells that were elicited by the priming. Therefore, while prolonging the lives of antigen-expressing DCs during priming and boosting leads to the strongest clonal expansion of antigen-specific $CD8^+$ T cells, it is during the boosting phase that this effect on DCs contributes most to T cell expansion.

The Bak and Bax siRNA technology can also be extended to the treatment of DCs *ex vivo* for subsequent *in vivo* use. As disclosed above, E7 peptide-pulsed DC-I cells transfected with Bak+Bax siRNA were more resistant to killing by E7-specific $CD8^+$ T cells than were DC-I cells transfected with control siRNA.

Furthermore, as described in the Examples below, vaccination with E7 peptide-pulsed DC-I cells transfected with Bak+Bax siRNA leads to significantly higher numbers of E7-specific $CD8^+$ T cells compared to vaccination with antigen -pulsed DC-I cells transfected with control siRNA. Thus, the potency of DC-based vaccines prepared *ex vivo* can be further enhanced by the specific targeting of key pro-apoptotic proteins, such as Bak and Bax, using siRNA.

In summary, the targeting of Bak+Bax siRNA with DNA vaccines (encoding antigen) to DCs *in vivo* represents an innovative approach to enhancing DNA vaccine potency. In addition, the use of siRNA alleviates safety concerns associated with the use of DNA vaccines encoding anti-apoptotic proteins. Not only does gene gun delivery of siRNA to DCs result in prolonged DC life, but it also avoids concerns for oncogenicity associated with DNA encoding anti-apoptotic proteins. Further safety is achieved by using detoxified (mutant) forms of the HPV antigens E7 or E6 as disclosed above. Indeed no gross anatomical or histological changes were observed in the vital organs of vaccinated mice compared to non-vaccinated mice, alleviating concerns about the induction of autoimmunity that as a sequela of prolonging DC life. Thus, the strategy of using siNA to silence pro-apoptotic proteins, as exemplified with siRNA targeting Bak+Bax useful in the clinical arena where enhanced DNA vaccine potency is a desirable goal in improving the immunologic control of cancer or infectious disease.

EXAMPLE 9

(Examples 9-15 incorporate by reference Peng S et al, Hum Gene Titer 16:584-93 (2005 May)

Transfected of Dendritic Cells with Bak/Bax siRNA Abolishes Expression of Bak and Bax Proteins

Western blot analysis was performed to examine in DC-I cells (a murine DC line) the effects of transfection with Bak/Bax siRNA on expression of Bak and Bax proteins. As shown in **Figure 21,**

lysates from DC-I cells transfected with Bak/Bax siRNA showed significant reduction in the expression of Bak and Bax proteins 24 and 48 hrs after transfection. In contrast, when transfection with control siRNA was done, the expression of Bak and Bax did not differ from that in non-transfected DC-lcells. Analysis of β-actin expression in transfected DCs confirmed that equal amounts of cell lysates had been loaded in all the Western blots. These results indicate that transfection of DC-I cells with Bak/Bax siRNA abolishes Bak and Bax protein expression during the intervals examined.

DC-I cells transfected with Bak and/or Bax siRNA can resist CTL-induced apoptosis. E7-loaded, siRNA-transfected, DC-I cells were incubated with an E7-specific $CD8^+$ T cell line. These DC-1 cells resisted killing by E7-specific $CD8^+$ T cells *in vitro*. Taken together, these results show that transfection of DC-I cells with Bak and/or Bax siRNA downregulates Bak and Bax protein expression, a consequence of which is resistance to apoptosis caused by activated antigen-specific $CD8^+$ T cells in DCs.

EXAMPLE 10

Vaccination with E7 peptide-loaded DCs transfected with Bak/Bax siRNA leads to a significant increase in E7-specific IFN-γ^+ $CD8^+$ T cell precursors

To determine whether vaccination with E7 peptide-loaded DCs transfected with Bak/Bax siRNA could enhance the generation of E7-specific IFN-γ^+ $CD8^+$ T cell precursors in mice, ICCS and flow-cytometry analysis was performed on spleen cells from mice vaccinated with the various DC-I cells. As shown in **Figures** 22-23, mice vaccinated with E7-loaded DCs transfected with Bak/Bax siRNA exhibited an ~5.4-fold increase in the number of E7-specific IFN-γ^+ $CD8^+$ T cells (655+21) compared to mice vaccinated with E7-loaded DCs transfected with control siRNA (121+5) (which were similar to the number of E7-specific $CD8^+$ T cells induced by E7-loaded DC-I that remained untransfected). Thus, administration of DCs that are transfected with Bak/Bax siRNA is markedly more immunogenic than the use of control DCs when measured by the number of E7-specific IFN-γ^+ $CD8^+$ T cells that are generated *in vivo*.

EXAMPLE 11

Vaccination with E7 Peptide-Loaded BM-DCs Transfected with Bak/Bax siRNA Increases E7-specific IFN-γ^+ $CD8^+$ T cell Precursors

It was important to determine if the Bak/Bax siRNA technology also works with a more "physiological" source of DCs, not derived from an immortalized cell line, since the former would be a more appropriate source of cells for clinical use. For this purpose bone marrow-derived DCs (BM-DCs) were tested - after loading with E7 peptide and transfection with either Bak/Bax siRNA or control siRNA. To determine E7-specific CD8+ T cell precursors in vaccinated mice, ICCS followed by flow

cytometry analysis was performed. As shown in **Figures** 24-25, mice vaccinated with E7-peptide-loaded BM-DCs transfected with Bak/Bax siRNA exhibited a ~2.2-fold increase in the number of E7-specific *WN-J*$^+$CD8$^+$ T cells (4706+78.5) compared to mice vaccinated with E7 peptide-loaded DCs transfected with control siRNA (2210+134.3) ($p<0.002$). Thus, the Bak/Bax siRNA technology can also be applied to BM-DCs to enhance their potency as immunogens.

EXAMPLE 12

Vaccination with E7-loaded DCs Transfected with Bak/Bax siRNA Generates Stronger Antitumor Effects than E7-loaded DCs Transfected with control siRNA

To determine whether the observed increase in the number of E7-specific CD8$^+$ T cell precursors translated into a stronger E7-specific antitumor effect, an *in vivo* tumor protection experiment was carried out using the TC-I system *(supra).* As shown in **Figure** 26, 100% of mice receiving E7 peptide-loaded DCs transfected with either control siRNA or Bak/Bax siRNA remained tumor-free for 30 days after a s.c. challenge with TC-I cells, whereas non-vaccinated mice developed tumors within 10 days of tumor challenge. Therefore, vaccination with E7 peptide-loaded DC-I transfected with either Bak/Bax siRNA or control siRNA elicited protective antitumor immunity against challenge by an E7-expressing tumor. The *in vivo* tumor protection model failed to distinguish between the use of Bak/Bax targeted and control siRNA.

To extend the comparison, an *in vivo* tumor trial was performed using a more stringent lung tumor metastasis model in which TC-I tumor cells were delivered i.v. Thus, mice were first challenged with the TC-I tumor cells i.v. (tail vein) followed by treatment with E7-peptide loaded DC-I cells transfected either with Bak/Bax siRNA or with control siRNA. Mice were sacrificed 28 days after the tumor challenge and the growth of pulmonary nodules was examined. As shown in **Figure 27,** mice treated with E7-peptide loaded DCs transfected with Bak/Bax siRNA demonstrated the lowest number of pulmonary nodules (2.2+0.84) compared to mice treated with E7-peptide loaded DCs transfected with control siRNA (24.8±5.89), or the naïve control group (103+12.29; /?<0.001; Student's t test). Thus, vaccination with E7-loaded DCs transfected with Bak/Bax siRNA generates a markedly better highly significant therapeutic effect than vaccination with E7-loaded DCs transfected with control siRNA.

EXAMPLE 13

E7 peptide-loaded DCs Transfected with Bak/Bax siRNA Survive Longer *In Vivo* than E7 Peptide-loaded DCs Transfected with Control siRNA.

To determine if transfection with Bak/Bax siRNA improves the survival of E7-peptide loaded DCs *in vivo,* two distinct groups of BM-DC cells loaded with carboxyfluorescein (CFSE)-labeled E7 peptide and transfected with different siRNAs were first created. E7 peptide-loaded BM-DCs

transfected with control siRNA were labeled with a higher concentration of CFSE (5μM), while Bak/Bax siRNA-transfected BM-DCs were labeled with a lower concentration of CFSE (0.5μM). The relative levels of CFSE in these two distinctly CFSE-labeled E7 peptide-loaded BM-DCs were characterized by flow cytometry **(Figure 28A).** Mice were then challenged with 10^6 E7-specific T cells/mouse i.v. Three days later, a mixture of $2.5xlO^5$ low CFSE-labeled BM-DCs and $2.5xlO^5$ of high CFSE-labeled BM-DCs were injected i.v. into each challenged mouse. Sixteen hours later, flow-cytometry analysis was performed to characterize the ratio of low CFSE-labeled BM-DCs to high CFSE-labeled BM-DCs using cells collected from the spleen and lungs of challenged mice. As shown in **Figure** 28B, a significantly higher number of low CFSE-labeled BM-DCs was observed (~3.7-fold), compared to the number of high-CFSE-labeled BM-DCs. These results show that transfection of E7 peptide-loaded BM-DCs with Bak/Bax siRNA can prolong DC life *in vivo,* and resulting in a higher number of E7-peptide loaded BM-DCs.

EXAMPLE 14

E7 Peptide-loaded DC-I Cells Transfected with Bak/Bax or Control siRNA express Similar Levels of CDllc, CD40. CD86. MHC I and MHC II.

The significant therapeutic effect generated by vaccination with E7-peptide-loaded DCs transfected with Bak/Bax siRNA could have been due to changes in the expression of molecules important for antigen presentation in DCs, such as CDl Ic, CD40, CD86, MHC I, and MHC H Flow cytometric analyses were done to determine the expression levels of these molecules in cells of an E7 peptide-loaded DC-I cell line transfected with Bak/Bax siRNA, control siRNA or in non-transfected DC-I cells. As shown in **Figure 29,** there was no significant change in the expression of any of the cell surface molecules evaluated among the E7 peptide-loaded DC-I cells. A similar study was done with BM-DCs. Again, no significant changes in the expression of these molecules were observed among the E7 peptide-loaded BM-DCs transfected with the various siRNA constructs (not shown). Taken together, these results indicated that the expression of CDl Ic, CD40, CD86, MHC class I, and MHC class II proteins on the surface of DCs that has been E7-peptide loaded were are not affected by Bak/Bax siRNA.

EXAMPLE 15

Discussion of Examples 9-14

This set of studies demonstrated that vaccination with E7 peptide-loaded DCs transfected with Bak/Bax siRNA generated enhanced E7-specific T cell-mediated immune responses and antitumor effects *in vivo.* Transfection of DCs with Bak/Bax siRNA inhibited apoptotic cell death of DCs mediated by T cells, leading to prolongation of DC survival and resulting in an improved DC-based vaccine.

Previous studies showed that DC life can be efficiently prolonged *in* vivo through transfection of DCs with DNA encoding antiapoptotic proteins (Kim, TW *et ah, J Clin Invest 112:109-17,* 2003b). This technique, however, has raised concerns regarding potential oncogenic transformation as a result of overexpression of these antiapoptotic proteins. Antiapoptotic proteins such as the Bcl-2 family are known to be over-expressed in some cancers and therefore have been implicated as contributors to cellular immortalization (Lebedeva, I., *Cancer Res.* 50:6052-60, 2000). The modification of DCs using siRNA targeting Bak and Bax proteins alleviates many of these concerns. Due to the transient nature of siRNA-mediated silencing of target genes as well as the fact that RNA-based strategies carry no concerns for integration and permanent genetic change, transfection of DCs with Bak/Bax siRNA represent a potentially safe and effective method for enhancing DC-based vaccine potency by prolonging DC life without risk of DC immortalization.

Results employing this DC-based vaccine prepared *ex vivo* using siRNA technology targeting Bak and Bax are consistent with results of modifying DCs using Bak/Bax siRNA vaccination *in vivo.* Examples 2-8 describe intradermal gene-gun co-administration of DNA encoding antigen with Bak/Bax siRNA to prolong the life of antigen -expressing DCs *in vivo.* Mice vaccinated with DNA coadministered with Bak/Bax siRNA manifest significantly enhanced antigen-specific $CD8^+$T cell-mediated immune responses and antitumor effects compared to mice vaccinated with DNA coadministered with control siRNA. Taken together, these results indicate that siRNA technology as described herein can be used to modify DCs either *ex vivo* or *in vivo* to improve vaccine potency.

Modification of a DC-based vaccine with Bak/Bax siRNA as well as siRNA targeting other key pro-apoptotic proteins will further enhance DC-based vaccine potency. Since Bak/Bax siRNA only affects the intrinsic granzyme B/perforin-mediated apoptotic pathway, a combination of siRNAs targeting key pro-apoptotic proteins in the intrinsic granzyme B/perforin pathway along with siRNAs targeting other key pro-apoptotic proteins in the extrinsic Fas-mediated apoptotic pathway will likely result in stronger resistance to killing of the transfected DCs by T cells *in vivo.* As discussed above, caspase-8, a caspase that induces the proteolysis of a cascade of effector caspases leading to apoptotic cell death, is an excellent candidate protein to target for RNAi. Other caspases involved in cell apoptosis that could serve as targets for siRNA include caspase 9 and caspases 3, 6, and 7. Thus, a DC-based vaccination strategy employing siRNAs targeting key pro-apoptotic proteins in both the intrinsic and extrinsic apoptotic pathways, for example, antigen-loaded DCs transfected with Bak/Bax siRNA and caspase-8 siRNA, are expected to result in even greater enhancement of DC resistance to endogenous T cell-mediated killing, and this will result in improved T cell immune response and antitumor effects *in vivo.*

In the present study, antigen was loaded onto DCs by pulsing DCs with antigenic peptides. This Bak/Bax siRNA technology could also be applied to DCs prepared through other antigen-loading strategies, including viral vector-mediated, protein-mediated, RNA-mediated, and DNA-mediated transfection strategies. Viral vector-mediated strategies show highly efficient transfection of DCs, but have a limited "life expectancy", whereas DNA-mediated strategies are easily prepared but have a lower transfection efficiency in DCs. Thus, both viral vector-mediated and DNA-mediated strategies to deliver antigens to DCs benefit from the use of Bak/Bax siRNA technology. It will be possible to further enhance the potency of DC-based vaccines through the combined use of Bak/Bax siRNA as an antiapoptotic strategy with other vaccine enhancement strategies, such as the intracellular targeting of antigen inside DCs using various IPPs for more efficient intracellular processing. According to the present invention DNA-mediated strategies of DC-based vaccination employ DCs transfected with Bak/Bax siRNA co-administered with DNA plasmids comprising a DNA sequence encoding an antigen peptide linked to DNA encoding an IPP such as HSP70. The IPP targets the antigen for intracellular processing within the DCs, thereby resulting in increased expression/presentation of the antigen on the DC surface, while transfection by Bak/Bax siRNA would prolong the life of the DCs. The combination of these effects will increase T cell activation and result in an enhanced antigen-specific immune response.

In summary, antigen-loaded DCs transfected with Bak/Bax siRNA as a DC-based vaccine strategy offers an effective and potentially safer approach for prolonging the life of DCs and increasing the potency of DC-based vaccines than transfection of DCs *in vivo* with DNA encoding antiapoptotic proteins. Administering antigen-peptide loaded DCs transfected with Bak/Bax siRNA prolongs the life of transfected DCs and enhances antigen-specific $CD8^+$ T cell activity, as well as eliciting strong antitumor effects *in vivo*. Thus, a DC-based vaccine strategy incorporating antigen-loaded DCs transfected with Bak/Bax siRNA shows potential is readily adaptable to clinical use with DC-based vaccines for the control of cancer and infectious disease.

The references cited above are all incorporated herein by reference, whether specifically incorporated or not. All publications, patents, patent applications, GenBank sequences and ATCC deposits, cited herein are hereby expressly incorporated by reference for all purposes. Citation of the documents herein is not intended as an admission that any of them is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents. Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

WHAT IS CLAIMED IS;

1. A nucleic acid composition useful as an immunogen, comprising a combination of:

(a) a first nucleic acid molecule comprising a first sequence encoding an epitope of an antigenic polypeptide or peptide; and optionally, linked to the first sequence, directly or via a linker, a second sequence that encodes an immunogenicity-potentiating polypeptide (IPP); and

(c) a second nucleic acid molecule the activity or expression of which stimulates development of an immune response to said epitope, which second nucleic molecule is (i) a siNA or (ii) DNA that encodes said siNA, wherein said siNA has a sequence that is sufficiently complementary to and thus targets the sequence of mRNA that encodes a pro-apoptotic protein expressed in a dendritic cell (DC), such that the activity or expression of said siNA in the cell results in inhibition of or loss of expression of said mRNA, resulting in inhibition of apoptosis and increased survival of DCs,

wherein the development of said immune response is stimulated.

2. The composition of claim 1 that includes said second nucleic acid sequence encoding said IPP which is fused in frame to said first sequence such that said first and said second sequence encode a fusion protein comprising said antigenic epitope and said IPP.

3. The composition of claim 1, wherein the IPP acts in potentiating an immune response by promoting:

(a) processing of the linked antigenic polypeptide via the MHC class I pathway or targeting of a cellular compartment that increases said processing;

(b) development, accumulation or activity of antigen presenting cells or targeting of antigen to compartments of said antigen presenting cells leading to enhanced antigen presentation;

(c) intercellular transport and spreading of the antigen; or

(d) any combination of (a)-(c).

4. The composition of claim 3 wherein the IPP is:

(a) the sorting signal of the lysosome-associated membrane protein type 1 (Sig/LAMP-1)

(b) a mycobacterial HSP70 polypeptide, the C-terminal domain thereof, or a functional homologue or derivative of said polypeptide or domain;

(c) a viral intercellular spreading protein selected from the group of herpes simplex virus-1 VP22 protein, Marek's disease virus UL49 protein or a functional homologue or derivative thereof;

(d) an endoplasmic reticulum chaperone polypeptide selected from the group of calreticulin or a domain thereof, ER60, GRP94, gp96, or a functional homologue or derivative thereof.

(e) domain II of *Pseudomonas* exotoxin ETA or a functional homologue or derivative thereof;

(f) a polypeptide that targets the centrosome compartment of a cell selected from γ-tubulin or a functional homologue or derivative thereof; or

(g) a polypeptide that stimulates DC precursors or activates DC activity selected from the group consisting of GM-CSF, Flt3-ligand extracellular domain, or a functional homologue or derivative thereof.

5. The composition of any of claims 1-4 wherein said pro-apoptotic protein is selected from the group consisting of one or more of (a) Bak, (b) Bax, (c) caspase-8, (d) caspase-9 and (e) caspase-3.

6. The composition of claim 5 wherein said anti-apoptotic protein, the encoding mRNA of which is targeted by said siNA, is Bak and/or Bax.

7. The composition of claim 5 wherein said siNA is an siRNA.

8. The composition of claim 6 wherein said siNA is an siRNA.

9. The composition of claim 8 wherein said siRNA targets SEQ ID NO:4 of Bak and/or SEQ ID NO:8 ofBax.

10. The composition of claim 9 wherein said siRNA is selected from the group consisting of:

(a) SEQ ID NO: 1/SEQ ID NO:2; and

(b) SEQ ID NO:5/SEQ ID NO:6.

11. The composition of claim 1 wherein the antigenic polypeptide or peptide comprises an epitope that binds to and is presented on surfaces of antigen-presenting cells by MHC class I proteins.

12. The composition of claim 5 wherein the antigenic polypeptide or peptide comprises an epitope that binds to and is presented on surfaces of antigen-presenting cells by MHC class I proteins.

13. The composition of claim 12 wherein the epitope is between about 8 and about 11 amino acid residues in length.

14. The composition of any of claims 1-4 wherein the antigenic polypeptide or peptide is:

(i) is derived from a pathogen selected from the group consisting of a mammalian cell, a microorganism or a virus;

(ii) cross-reacts with an antigen of the pathogen; or

(iii) is expressed on the surface of a pathogenic cell.

15. The composition of claim 14 wherein the virus is a human papilloma virus.

16. The composition of claim 14, wherein the antigen is an HPV-16 E7, E7(detox), E6 or E6(detox) polypeptide or peptide.

17. The composition of claim 14 wherein the pathogen is a bacterium.

18. The composition of claim 14, wherein the antigenic polypeptide or peptide is a tumor-specific or tumor-associated antigen.

19. The composition of claim 1 wherein the first nucleic acid molecule is an expression vector comprising a promoter operatively linked to said first and/or said second sequence.

20. The composition of claim 19, wherein the promoter is one which is expressed in an antigen presenting cell (APC).

21. The composition of claim 20, wherein the APC is a DC.

22. Particles comprising a material suitable for introduction into a cell or an animal by particle bombardment to which particles is bound the composition of any of claims 1-4.

23. Particles comprising a material is suitable for introduction into a cell or an animal by particle bombardment to which particles is bound the composition of claim 5.

24. Particles comprising a material is suitable for introduction into a cell or an animal by particle bombardment to which particles is bound the composition of claim 6.

25. Particles comprising a material is suitable for introduction into a cell or an animal by particle bombardment to which particles is bound the composition of claim 9.

26. Particles comprising a material is suitable for introduction into a cell or an animal by particle bombardment to which particles is bound the composition of claim 10.

27. Particles comprising a material is suitable for introduction into a cell or an animal by particle bombardment to which particles is bound the composition of claim 16.

28. A combination of first and second particles each comprising a material is suitable for introduction into a cell or an animal by particle bombardment, and to which particles is bound the composition of claim 1, wherein

(a) the first nucleic acid molecules are bound to said first particles; and

(b) the second nucleic acids are bound to said second particles.

29. The particles of any of claims 22-28 which are gold particles.

30. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the composition of any of claims 1-4 and a pharmaceutically acceptable carrier or excipient.

31. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the composition of claim 5 and a pharmaceutically acceptable carrier or excipient.

32. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the composition of claim 6 and a pharmaceutically acceptable carrier or excipient.

33. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the composition of claim 9 and a pharmaceutically acceptable carrier or excipient.

34. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the composition of claim 10 and a pharmaceutically acceptable carrier or excipient.

35. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the composition of claim 16 and a pharmaceutically acceptable carrier or excipient.

36. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the particles of any of claims claim 22-28, and a pharmaceutically acceptable carrier or excipient.

37. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the composition of any of claims 1-4, thereby inducing or enhancing the antigen specific immune response.

38. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the composition of claim 5, thereby inducing or enhancing the antigen specific immune response.

39. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the composition of claim 6, thereby inducing or enhancing the antigen specific immune response.

40. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the composition of claim 9, thereby inducing or enhancing the antigen specific immune response.

41. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the composition of claim 10, thereby inducing or enhancing the antigen specific immune response.

42. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the composition of claim 16, thereby inducing or enhancing the antigen specific immune response.

43. A method of inducing or enhancing an antigen specific immune response in a subject, comprising administering to the subject an effective amount of the particles of claim 22, thereby inducing or enhancing the antigen specific immune response.

44. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the particles of claim 23, thereby inducing or enhancing the antigen specific immune response.

45. A method of inducing or enhancing an antigen specific immune response in a subject, comprising administering to the subject an effective amount of the particles of claim 24, thereby inducing or enhancing the antigen specific immune response.

46. A method of inducing or enhancing an antigen specific immune response in a subject, comprising administering to the subject an effective amount of the particles of claim 25, thereby inducing or enhancing the antigen specific immune response.

47. A method of inducing or enhancing an antigen specific immune response in a subject, comprising administering to the subject an effective amount of the particles of claim 26, thereby inducing or enhancing the antigen specific immune response.

48. A method of inducing or enhancing an antigen specific immune response in a subject, comprising administering to the subject an effective amount of the particles of claim 27, thereby inducing or enhancing the antigen specific immune response.

49. A method of inducing or enhancing an antigen specific immune response in a subject, comprising administering to the subject an effective amount of the particles of claim 28, thereby inducing or enhancing the antigen specific immune response.

50. The method of claim 37, wherein the antigen specific immune response is mediated at least in part by $CD8^+$ cytotoxic T lymphocytes (CTL).

51. The method of claim 40, wherein the antigen specific immune response is mediated at least in part by $CD8^+$ cytotoxic T lymphocytes (CTL).

52. The method of claim 42, wherein the antigen specific immune response is mediated at least in part by $CD8^+$ cytotoxic T lymphocytes (CTL).

53. The method of claim 37, wherein the composition is administered to a human.

54. The method of claim 40, wherein the particles are administered to a human.

55. The method of claim 42, wherein the particles are administered to a human.

56. The method of claims 37, wherein the composition is administered intradermally by particle bombardment.

57. The method of claims 40, wherein the particles are administered intradermally by particle bombardment.

58. The method of claims 42, wherein the particles are administered intradermally by particle bombardment.

59. The method of claim 37 wherein the composition is administered intratumorally or peritumorally.

60. A method of increasing the numbers of $CD8^+$ CTLs specific for a selected desired antigen in a subject comprising administering an effective amount of the composition of any of claims 1-4 wherein the antigenic peptide or polypeptide comprises an epitope that binds to and is presented on surfaces of antigen-presenting cells by MHC class I proteins, thereby increasing the numbers of antigen-specific $CD8^+$ CTLs.

61. A method of increasing the numbers of $CD8^+$ CTLs specific for a selected desired antigen in a subject comprising administering an effective amount of the composition of claim 5, wherein the antigenic peptide or polypeptide comprises an epitope that binds to and is presented on surfaces of antigen-presenting cells by MHC class I proteins, thereby increasing the numbers of antigen-specific $CD8^+$ CTLs.

62. A method of increasing the numbers of $CD8^+$ CTLs specific for a selected desired antigen in a subject comprising administering an effective amount of the composition of claim 9, wherein the antigenic peptide or polypeptide comprises an epitope that binds to and is presented on surfaces of antigen-presenting cells by MHC class I proteins, thereby increasing the numbers of antigen-specific $CD8^+$ CTLs.

63. A method of increasing the numbers of $CD8^+$ CTLs specific for a selected desired antigen in a subject comprising administering an effective amount of the composition of claim 16, wherein the antigenic peptide or polypeptide comprises an epitope that binds to and is presented on surfaces of antigen-presenting cells by MHC class I proteins, thereby increasing the numbers of antigen-specific $CD8^+$ CTLs.

64. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of the composition of any of claims 1-4, wherein the antigenic epitopes are those expressed by the tumor or ones cross reactive with those expressed by the tumor, thereby inhibiting growth of the tumor.

65. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of the composition of claim 5, wherein the antigenic epitopes are those expressed by the tumor or ones cross reactive with those expressed by the tumor, thereby inhibiting growth of the tumor.

66. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of the composition of claim 9, wherein the antigenic epitopes are those expressed by the tumor or ones cross reactive with those expressed by the tumor, thereby inhibiting growth of the tumor.

67. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of the composition of claim 16, wherein the antigenic epitopes are those expressed by the tumor or ones cross reactive with those expressed by the tumor, thereby inhibiting growth of the tumor.

68. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of the particles of any claim 22, wherein the antigenic epitopes are those expressed by the tumor or ones cross reactive with those expressed by the tumor, thereby inhibiting growth of the tumor.

69. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of the particles of any claim 23, wherein the antigenic epitopes are those expressed by the tumor or ones cross reactive with those expressed by the tumor, thereby inhibiting growth of the tumor.

70. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of the particles of any claim 25, wherein the antigenic epitopes are those expressed by the tumor or ones cross reactive with those expressed by the tumor, thereby inhibiting growth of the tumor.

71. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of the particles of any claim 27, wherein the antigenic epitopes are those expressed by the tumor or ones cross reactive with those expressed by the tumor, thereby inhibiting growth of the tumor.

72. An immunogenic cellular composition, comprising dendritic cells (DCs) which have been modified by:

(a) loading the DCs with an antigen so that the antigen is expressed on the DC surface, or transducing or transfecting the DCs with DNA that encodes an antigen fused to an IPP; and

(b) transfecting the DCs with a nucleic acid molecule that is (i) a siNA or (ii) DNA that encodes said siNA, wherein said siNA has a sequence that is sufficiently complementary to and thus targets the sequence of mRNA that encodes a pro-apoptotic protein expressed in the DC, such that expression or activity said siNA in the cell results in diminution or loss of expression of said mRNA, resulting in inhibition of apoptosis and prolonged survival of the DC.

73. The composition of claims 72 wherein said pro-apoptotic protein is selected from the group consisting one or more of (a) Bak, (b) Bax, (c) caspase-8, (d) caspase-9 and (e) caspase-3.

74. The composition of claim 73 wherein said anti-apoptotic protein, the encoding mRNA of which is targeted by said siNA, is Bak and/or Bax.

75. The composition of claim 72 wherein said siNA is an siRNA.

76. The composition of claim 73 wherein said siNA is an siRNA.

77. The composition of claim 74 wherein said siNA is an siRNA.

78. The composition of claim 75 wherein said siRNA targets SEQ ID NO:4 of Bak and/or SEQ ID NO:8 ofBax.

79. The composition of claim 78 wherein said siRNA is selected from the group consisting of:

(a) SEQ ID NO: 1/SEQ ID NO:2; and

(b) SEQ ID NO:5/SEQ ID NO:6.

80. The composition of claim 76 wherein said siRNA targets SEQ ID NO:4 of Bak and/or SEQ ID NO:8 ofBax.

81. The composition of claim 80 wherein said siRNA is selected from the group consisting of:

(a) SEQ ID NO: 1/SEQ ID NO:2; and

(b) SEQ ID NO:5/SEQ ID NO:6.

82. The composition of claim 77 wherein said siRNA targets SEQ ID NO:4 of Bak and/or SEQ IDNO:8 ofBax.

83. The composition of claim 82 wherein said siRNA is selected from the group consisting of:

(a) SEQ ID NO: 1/SEQ ID NO:2; and

(b) SEQ ID NO:5/SEQ ID NO:6.

84. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the composition of any of claims 72-83 and a pharmaceutically acceptable carrier or excipient.

85. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the composition of any of claims 72-83, thereby inducing or enhancing the antigen specific immune response.

86. A method of increasing the numbers of $CD8^+$ CTLs specific for a selected desired antigen in a subject comprising administering an effective amount of the composition of any of claims 72-83 wherein the loaded antigen or the antigen expressed from said transduced DNA comprises an epitope that binds to and is presented on the DC surface by MHC class I proteins, thereby increasing the numbers of antigen-specific $CD8^+$ CTLs.

87. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of the composition of any of claims 72-83, wherein the antigenic epitopes are those expressed by the tumor or ones cross reactive with those expressed by the tumor, thereby inhibiting growth of the tumor.

88. Use of a nucleic acid composition as defined in any of claims 1-21 or particles as defined in any of claims 22-29, or a cellular composition as defined in any of claims 78-83 in the manufacture of a medicament for inducing or enhancing an antigen specific immune response in a subject.

89. Use of a nucleic acid composition as defined in any of claims 1-21 or particles as defined in any of claims 22-29, or a cellular composition as defined in any of claims 78-83 in the manufacture of a medicament such as a vaccine, for inducing an immune response in a subject.

90. Use of a nucleic acid composition as defined in any of claims 1-21 or particles as defined in any of claims 22-29, or a cellular composition as defined in any of claims 78-83 in the manufacture of a medicament for inhibiting the growth of a tumor or treating cancer in a subject wherein the antigenic epitopes are those expressed by the tumor or ones cross-reactive with those expressed by the tumor.

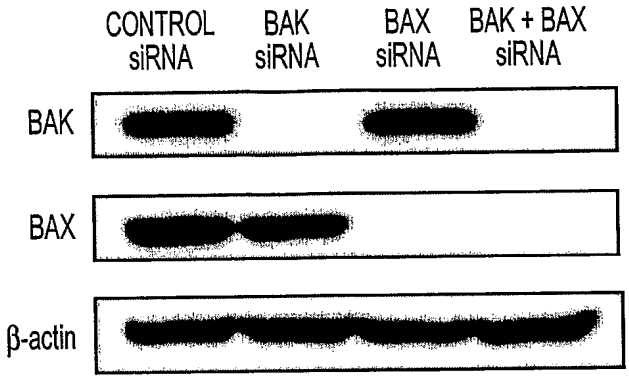
CONTROL siRNA
BAK siRNA
BAX siRNA
BAK + BAX siRNA
BAK
BAX
β-actin

FIG. 1

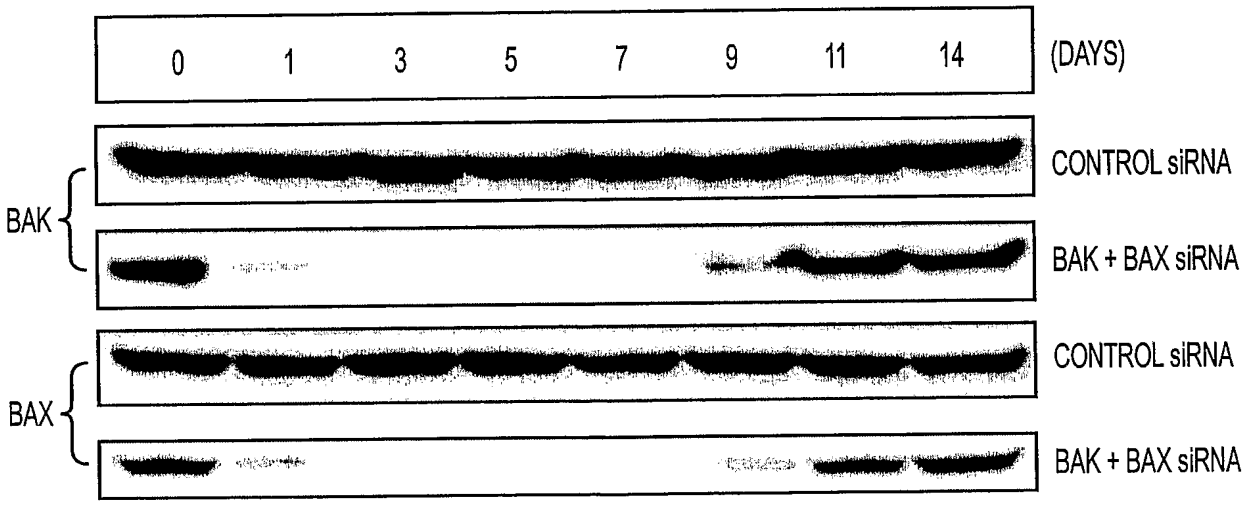
0
1
3
5
7
9
11
14
(DAYS)
BAK
BAX
CONTROL siRNA
BAK + BAX siRNA
CONTROL siRNA
BAK + BAX siRNA

FIG. 2

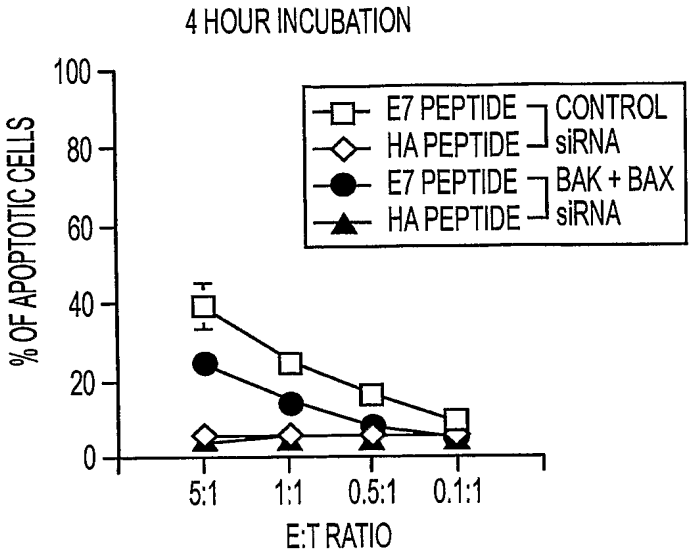
4 HOUR INCUBATION
% OF APOPTOTIC CELLS
100
80
60
40
20
0
5:1
1:1
0.5:1
0.1:1
E:T RATIO
E7 PEPTIDE
HA PEPTIDE
CONTROL siRNA
E7 PEPTIDE
HA PEPTIDE
BAK + BAX siRNA

FIG. 3A

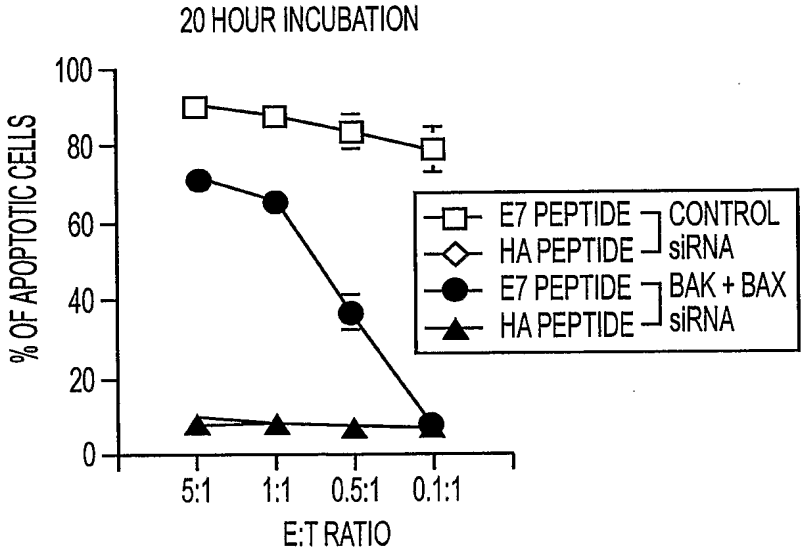
20 HOUR INCUBATION
% OF APOPTOTIC CELLS
100
80
60
40
20
0
5:1
1:1
0.5:1
0.1:1
E:T RATIO
E7 PEPTIDE
HA PEPTIDE
CONTROL siRNA
E7 PEPTIDE
HA PEPTIDE
BAK + BAX siRNA

FIG. 3B

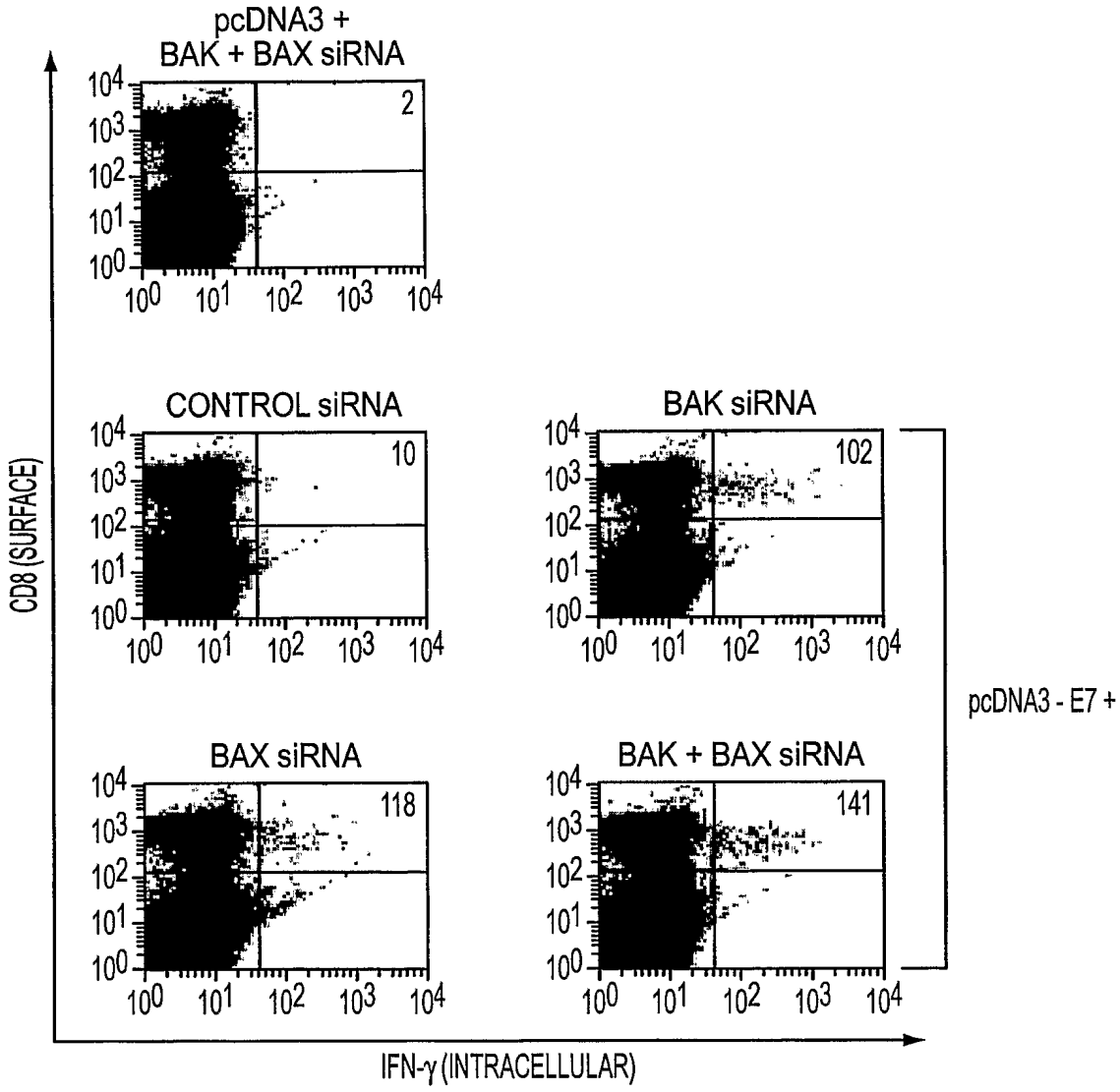
pcDNA3 +
BAK + BAX siRNA
2
CONTROL siRNA
10
BAK siRNA
102
BAX siRNA
118
BAK + BAX siRNA
141
pcDNA3 - E7 +
CD8 (SURFACE)
IFN-γ (INTRACELLULAR)

FIG. 4

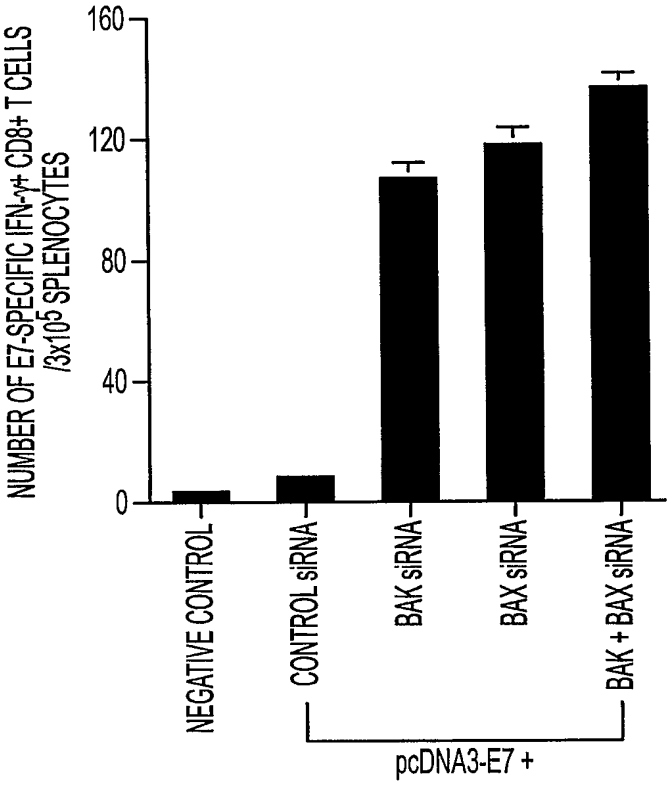
NUMBER OF E7-SPECIFIC IFN-γ+ CD8+ T CELLS /3x10^5 SPLENOCYTES
160
120
80
40
0
NEGATIVE CONTROL
CONTROL siRNA
BAK siRNA
BAX siRNA
BAK + BAX siRNA
pcDNA3-E7 +

FIG. 5

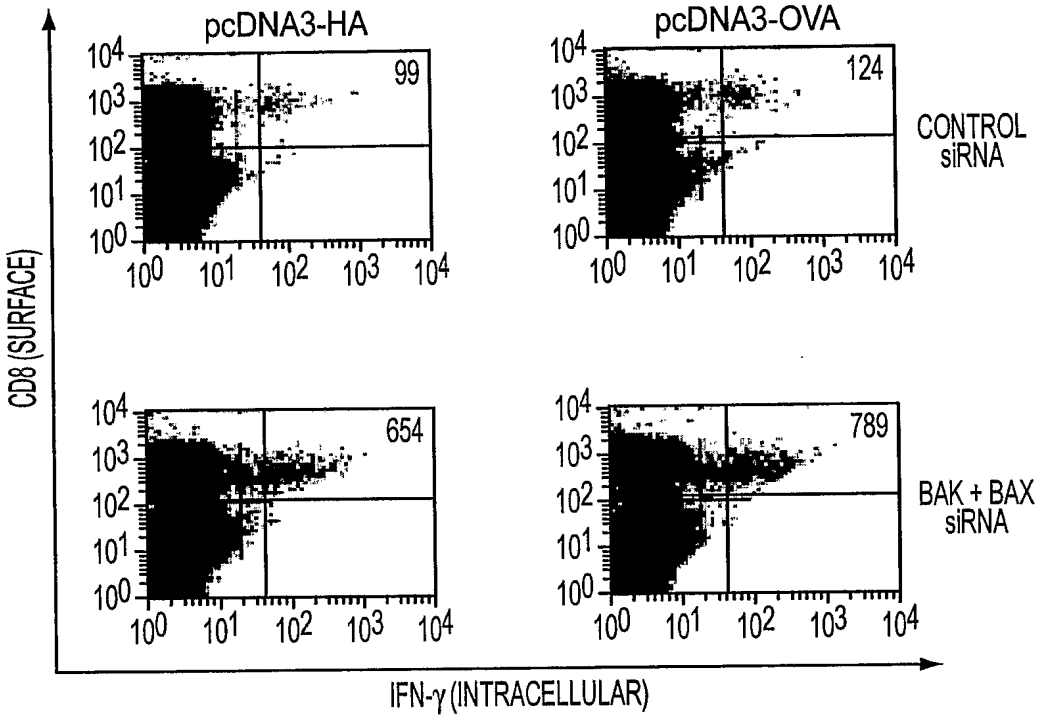
pcDNA3-HA
pcDNA3-OVA
99
124
654
789
CONTROL siRNA
BAK + BAX siRNA
CD8 (SURFACE)
IFN-γ (INTRACELLULAR)

FIG. 6

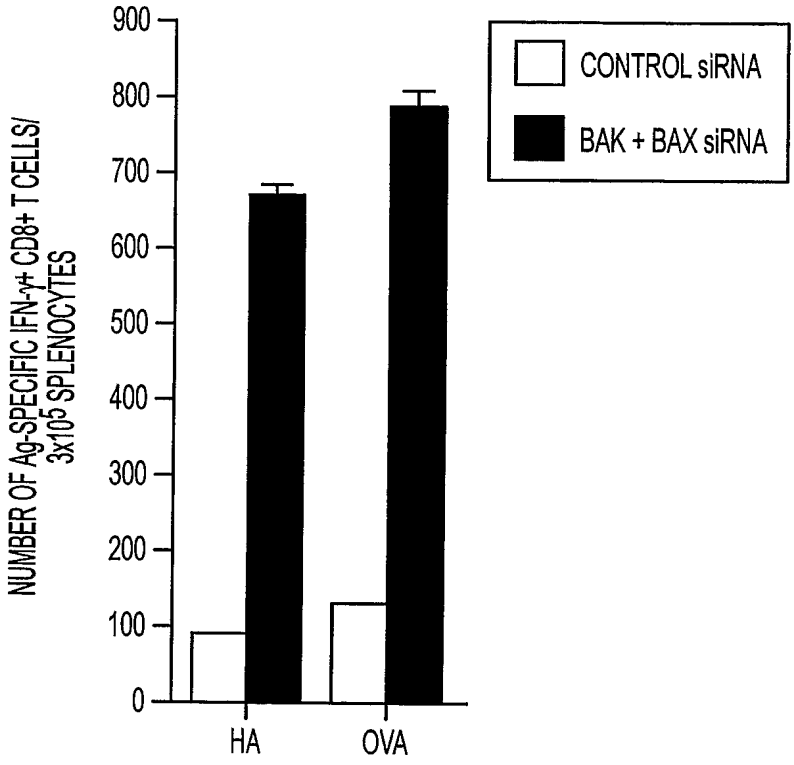
900
800
700
600
500
400
300
200
100
0
NUMBER OF Ag-SPECIFIC IFN-γ+ CD8+ T CELLS/ 3x10^5 SPLENOCYTES
HA
OVA
CONTROL siRNA
BAK + BAX siRNA

FIG. 7

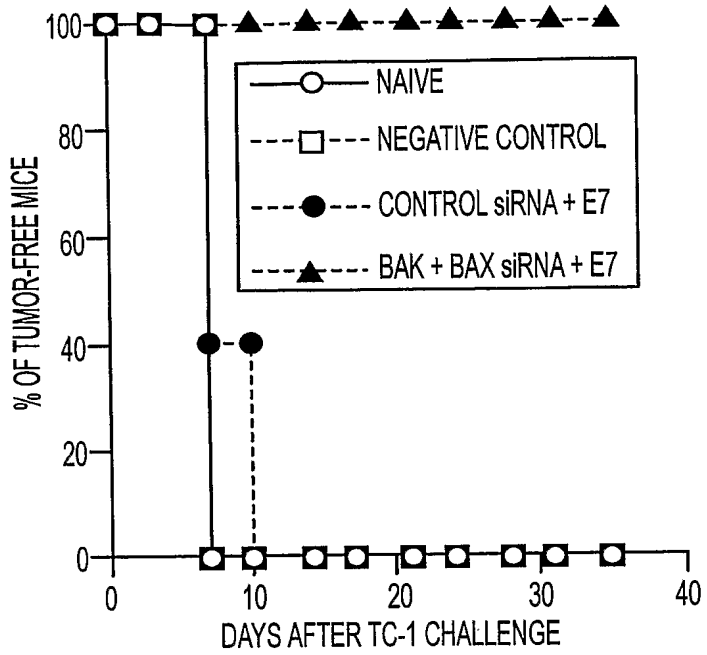
100
80
60
40
20
0
% OF TUMOR-FREE MICE
0
10
20
30
40
DAYS AFTER TC-1 CHALLENGE
NAIVE
NEGATIVE CONTROL
CONTROL siRNA + E7
BAK + BAX siRNA + E7

FIG. 8

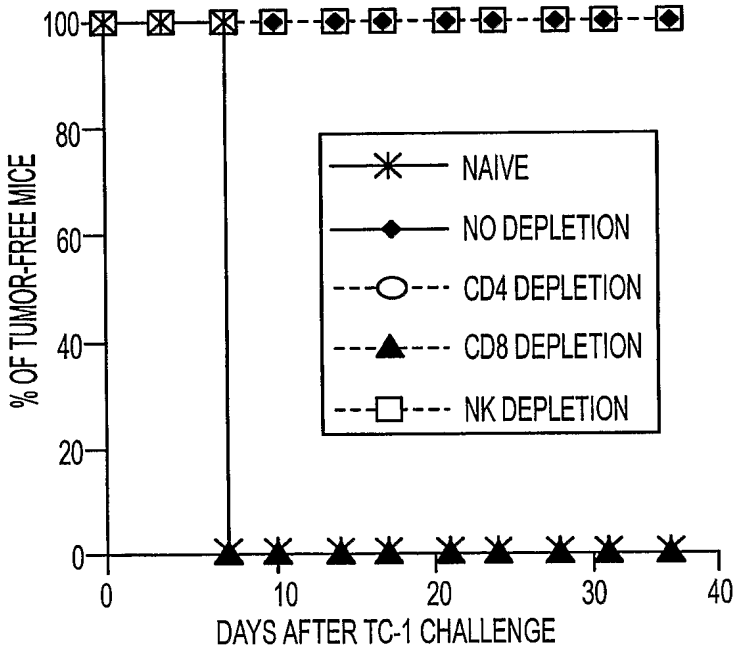
100
80
60
40
20
0
% OF TUMOR-FREE MICE
0
10
20
30
40
DAYS AFTER TC-1 CHALLENGE
NAIVE
NO DEPLETION
CD4 DEPLETION
CD8 DEPLETION
NK DEPLETION

FIG. 9

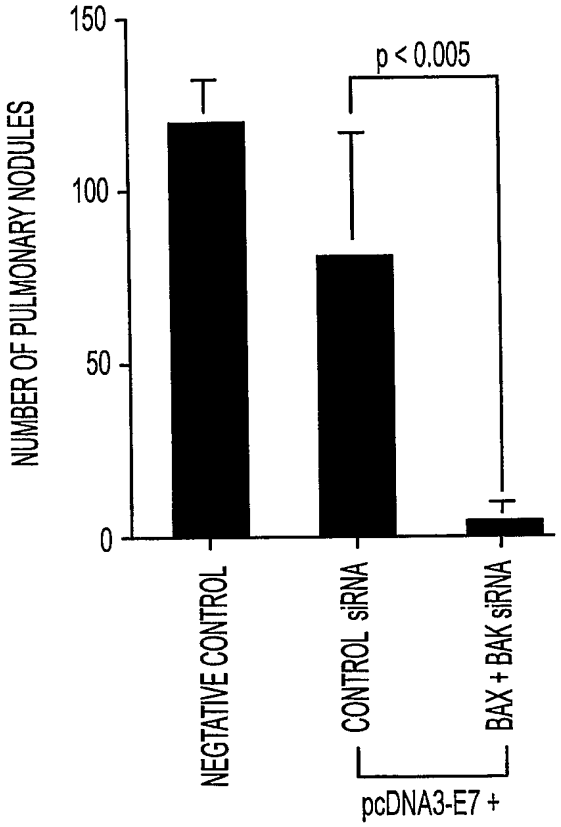
150
100
50
0
NUMBER OF PULMONARY NODULES
p < 0.005
NEGTATIVE CONTROL
CONTROL siRNA
BAX + BAK siRNA
pcDNA3-E7 +

FIG. 10

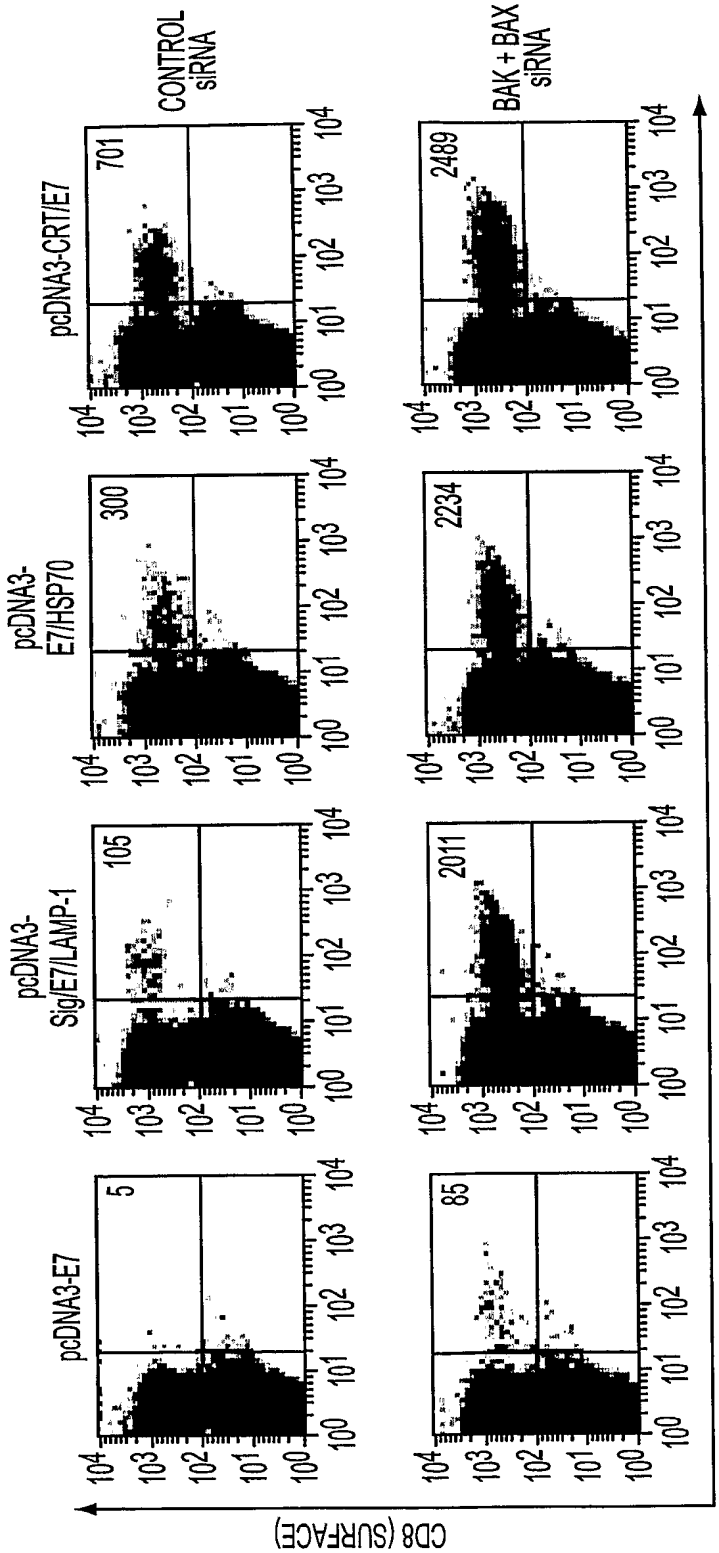
CONTROL siRNA
BAK + BAX siRNA
pcDNA3-CRT/E7
pcDNA3-E7/HSP70
pcDNA3-Sig/E7/LAMP-1
pcDNA3-E7
701
300
105
5
2489
2234
2011
85
CD8 (SURFACE)
IFN-γ (INTRACELLULAR)

FIG. 11

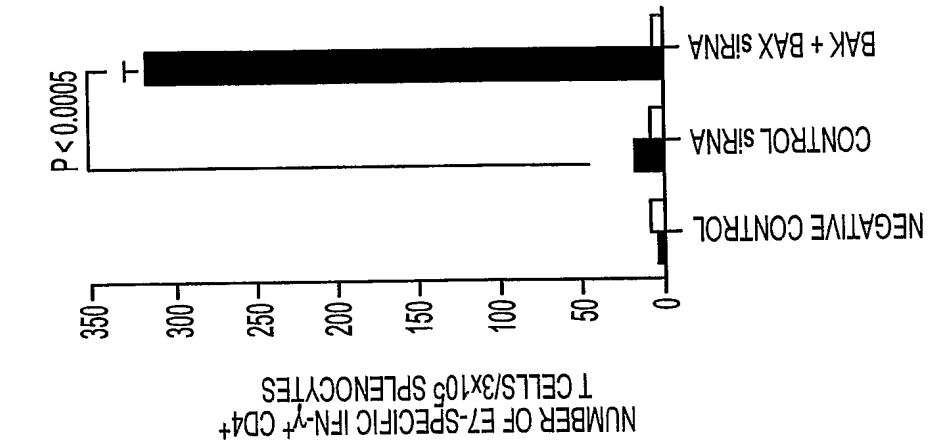
NUMBER OF E7-SPECIFIC IFN-γ+ CD4+
T CELLS/3x10⁵ SPLENOCYTES
0
50
100
150
200
250
300
350
NEGATIVE CONTROL
CONTROL siRNA
BAK + BAX siRNA
P < 0.0005

FIG. 13

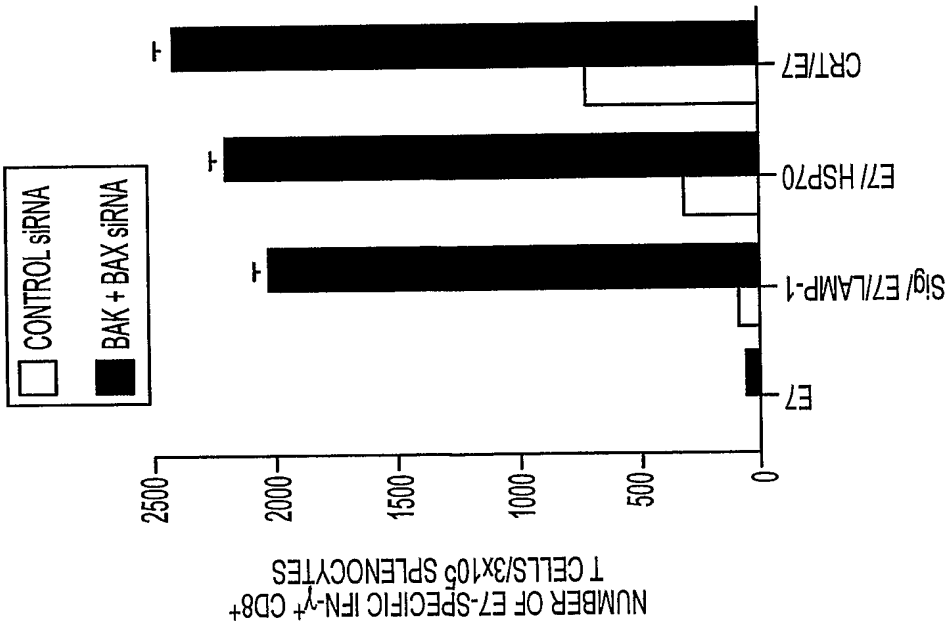
NUMBER OF E7-SPECIFIC IFN-γ+ CD8+
T CELLS/3x10⁵ SPLENOCYTES
0
500
1000
1500
2000
2500
E7
Sig/ E7/LAMP-1
E7/ HSP70
CRT/E7
BAK + BAX siRNA
CONTROL siRNA

FIG. 12

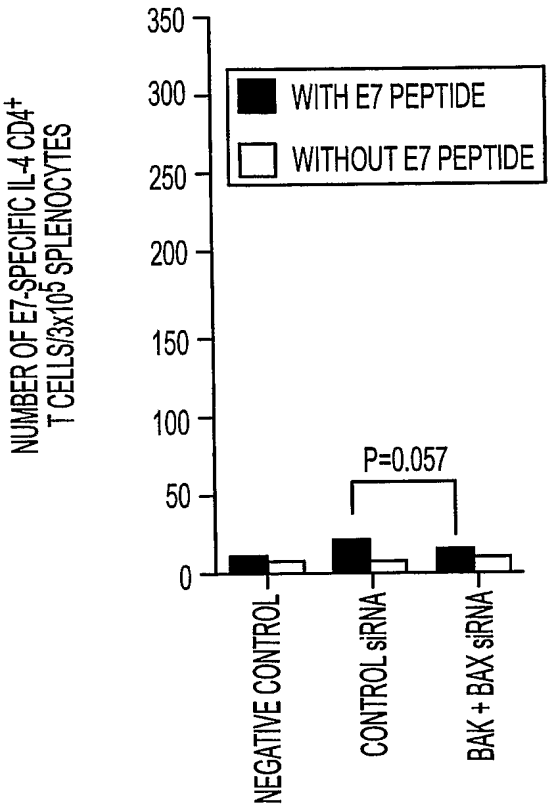
NUMBER OF E7-SPECIFIC IL-4 CD4+ T CELLS/3x10^5 SPLENOCYTES
350
300
250
200
150
100
50
0
WITH E7 PEPTIDE
WITHOUT E7 PEPTIDE
P=0.057
NEGATIVE CONTROL
CONTROL siRNA
BAK + BAX siRNA

FIG. 14

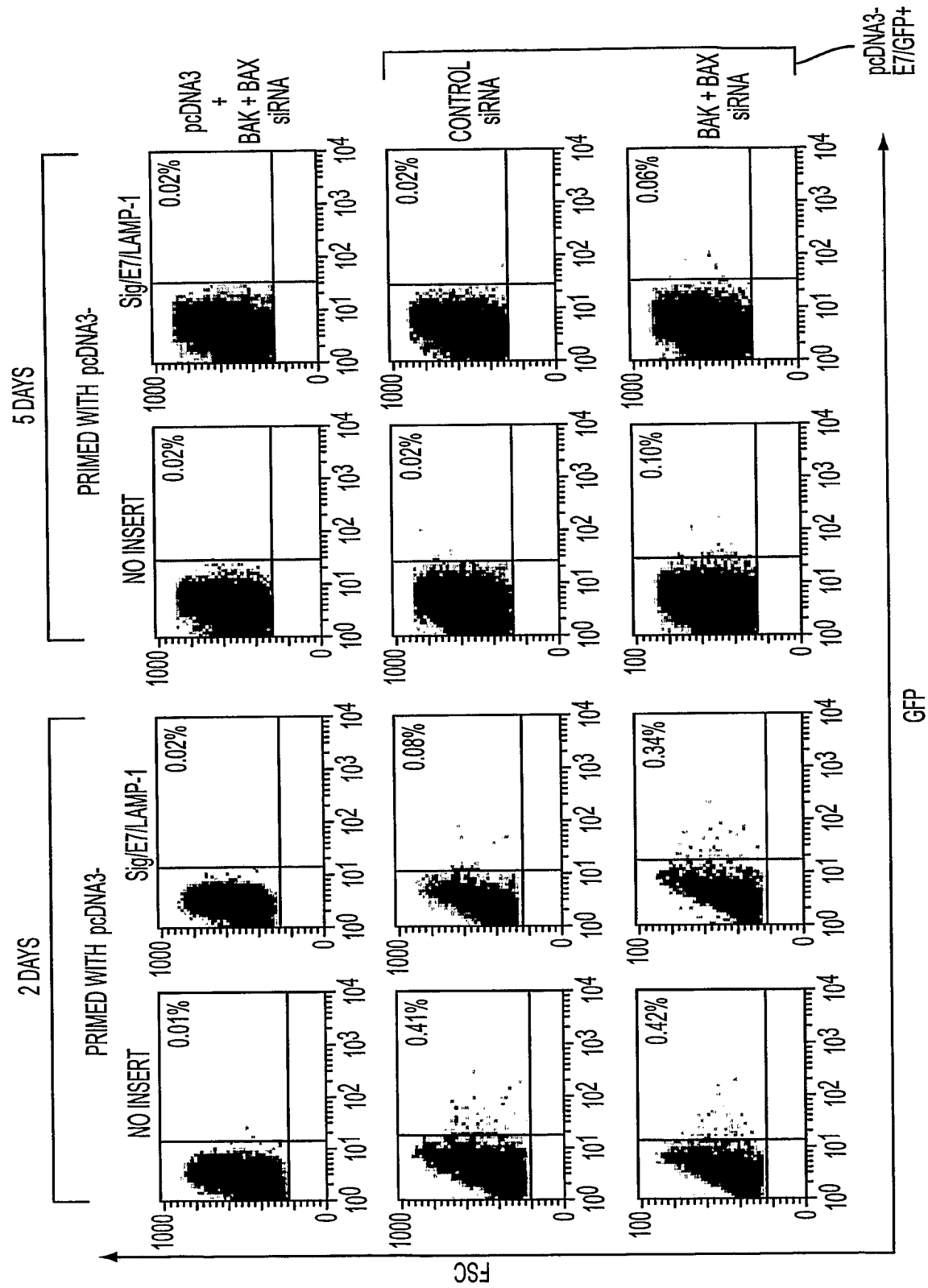
2 DAYS
5 DAYS
PRIMED WITH pcDNA3-
NO INSERT
Sig/E7/LAMP-1
pcDNA3 + BAK + BAX siRNA
CONTROL siRNA
BAK + BAX siRNA
pcDNA3-E7/GFP+
FSC
GFP
0.01%
0.02%
0.41%
0.08%
0.42%
0.34%
0.10%
0.06%

FIG. 15

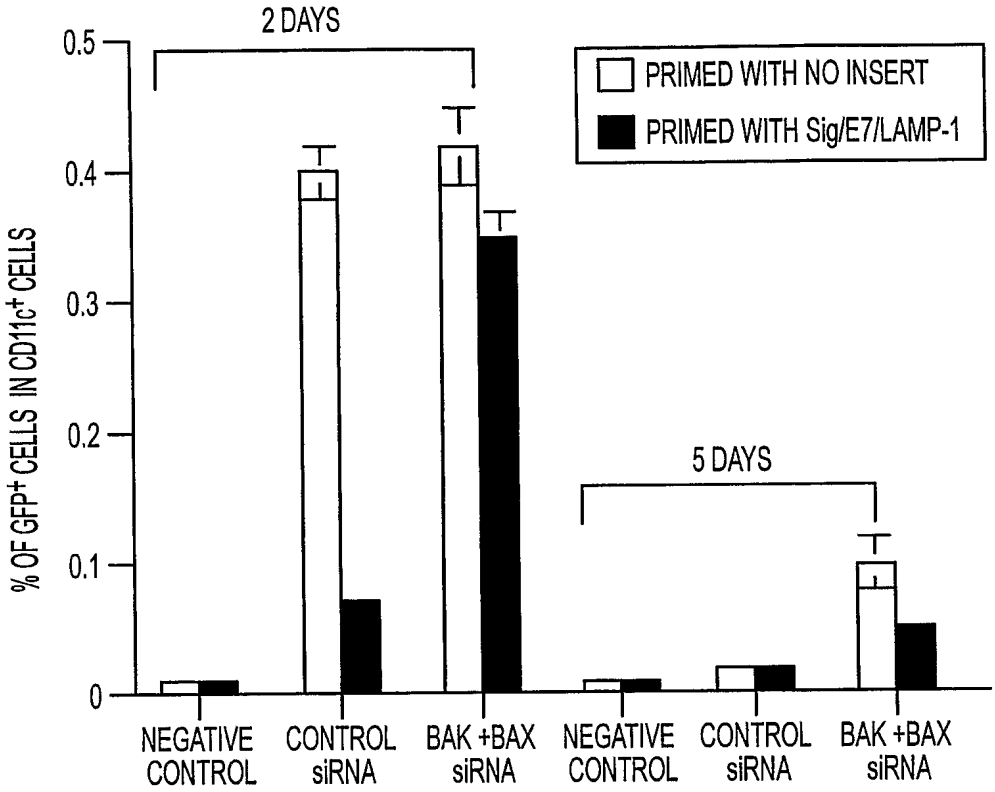
2 DAYS
5 DAYS
PRIMED WITH NO INSERT
PRIMED WITH Sig/E7/LAMP-1
% OF GFP+ CELLS IN CD11c+ CELLS
0.5
0.4
0.3
0.2
0.1
0
NEGATIVE CONTROL
CONTROL siRNA
BAK +BAX siRNA
NEGATIVE CONTROL
CONTROL siRNA
BAK +BAX siRNA

FIG. 16

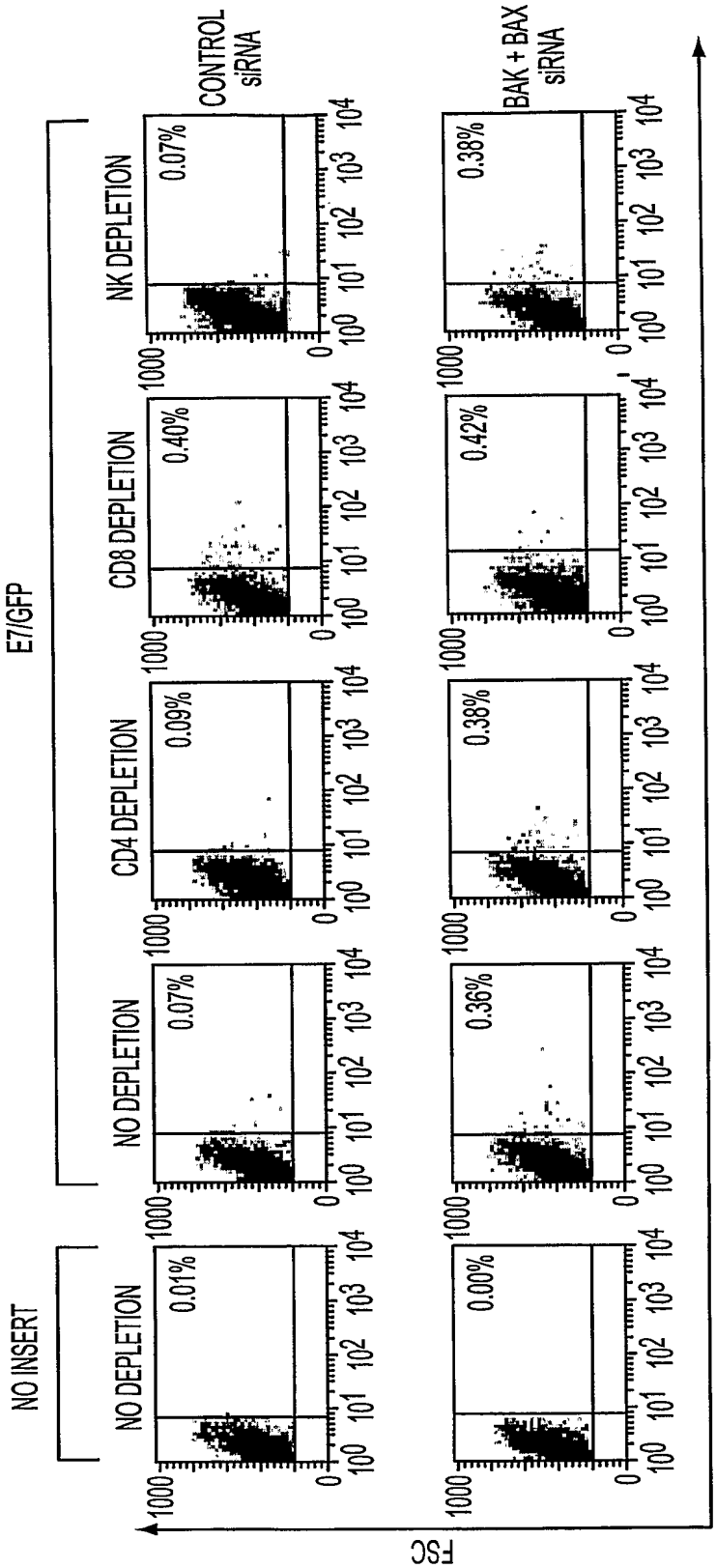
NO INSERT
E7/GFP
NO DEPLETION
CD4 DEPLETION
CD8 DEPLETION
NK DEPLETION
CONTROL siRNA
BAK + BAX siRNA
0.01%
0.07%
0.09%
0.40%
0.07%
0.00%
0.36%
0.38%
0.42%
0.38%
FSC
GFP

FIG. 17

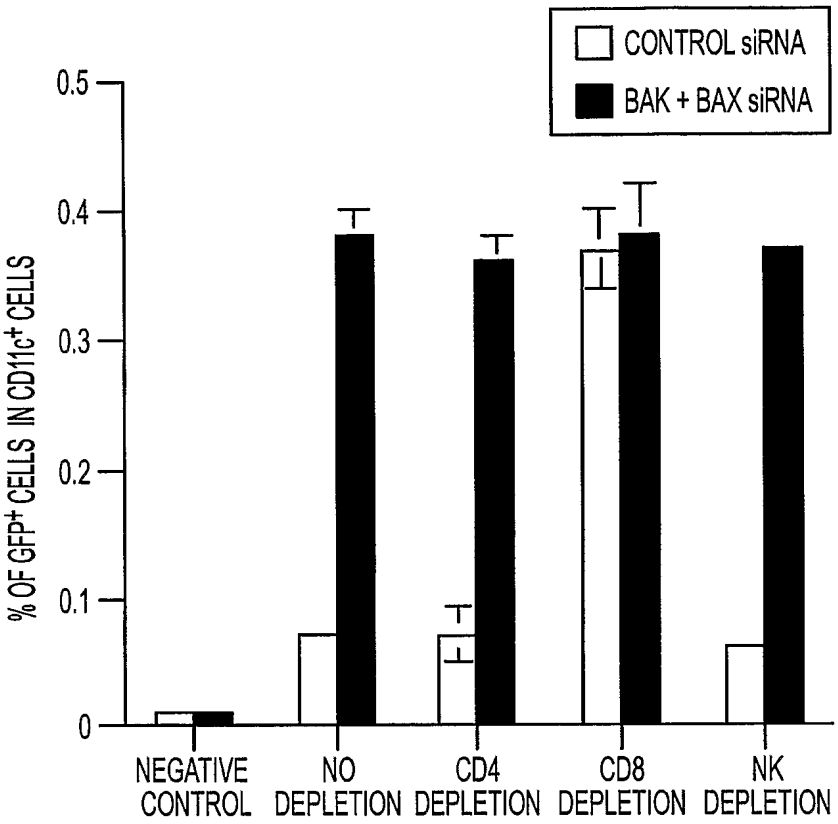
CONTROL siRNA
BAK + BAX siRNA
% OF GFP+ CELLS IN CD11c+ CELLS
0.5
0.4
0.3
0.2
0.1
0
NEGATIVE CONTROL
NO DEPLETION
CD4 DEPLETION
CD8 DEPLETION
NK DEPLETION

FIG. 18

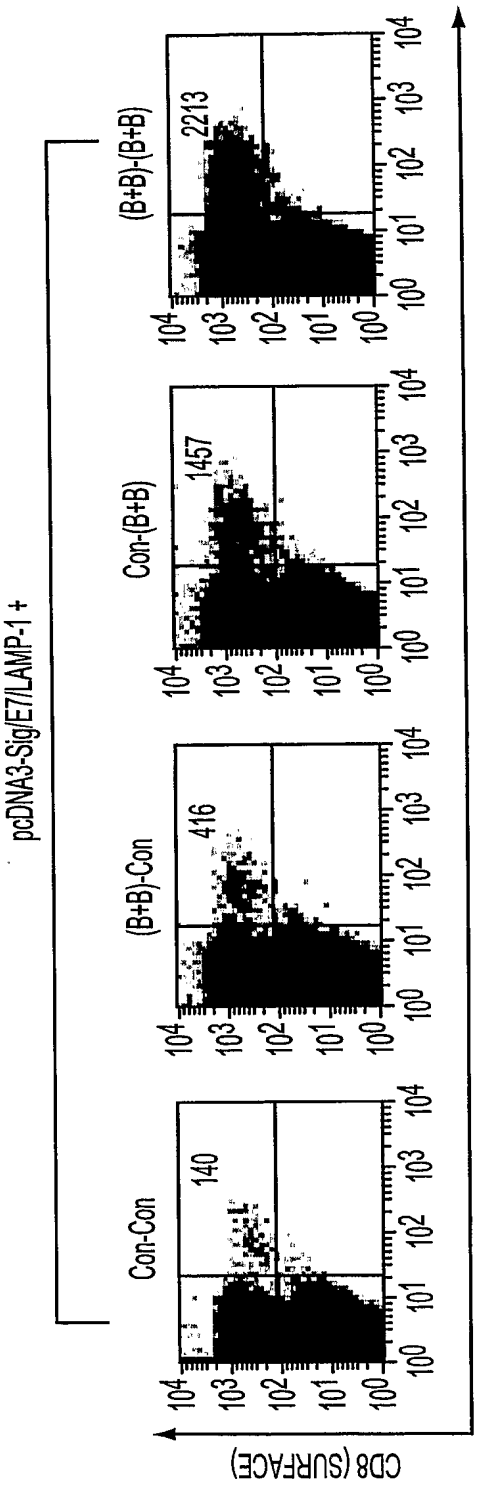
pcDNA3-Sig/E7/LAMP-1 +
Con-Con
140
(B+B)-Con
416
Con-(B+B)
1457
(B+B)-(B+B)
2213
CD8 (SURFACE)
IFN-γ (INTRACELLULAR)

FIG. 19

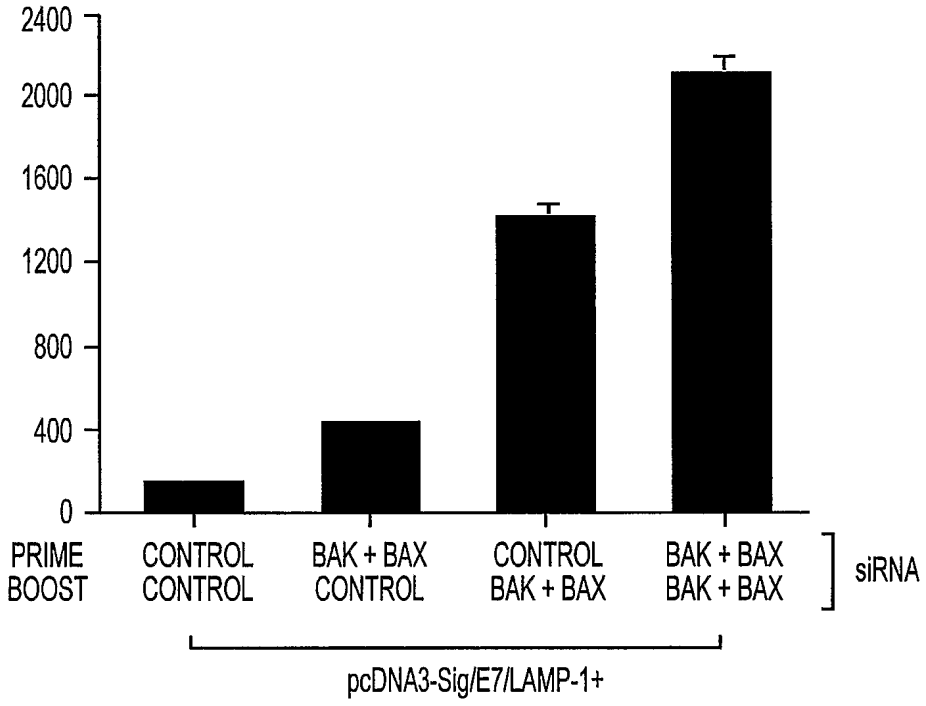
2400
2000
1600
1200
800
400
0
PRIME
BOOST
CONTROL
CONTROL
BAK + BAX
CONTROL
CONTROL
BAK + BAX
BAK + BAX
BAK + BAX
siRNA
pcDNA3-Sig/E7/LAMP-1+

FIG. 20

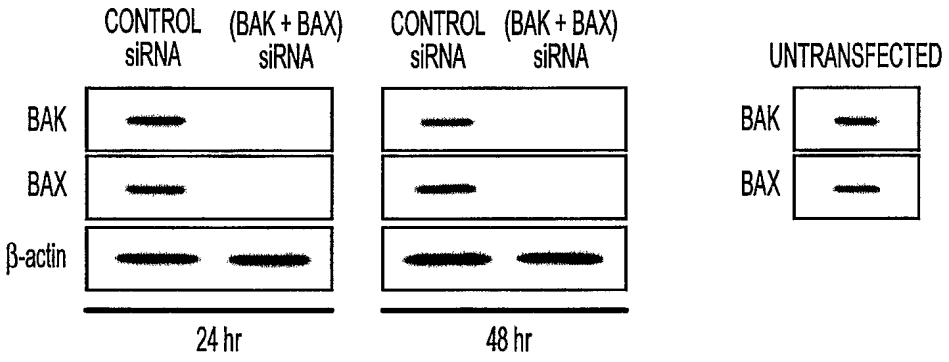
CONTROL siRNA
(BAK + BAX) siRNA
CONTROL siRNA
(BAK + BAX) siRNA
UNTRANSFECTED
BAK
BAX
β-actin
BAK
BAX
24 hr
48 hr

FIG. 21

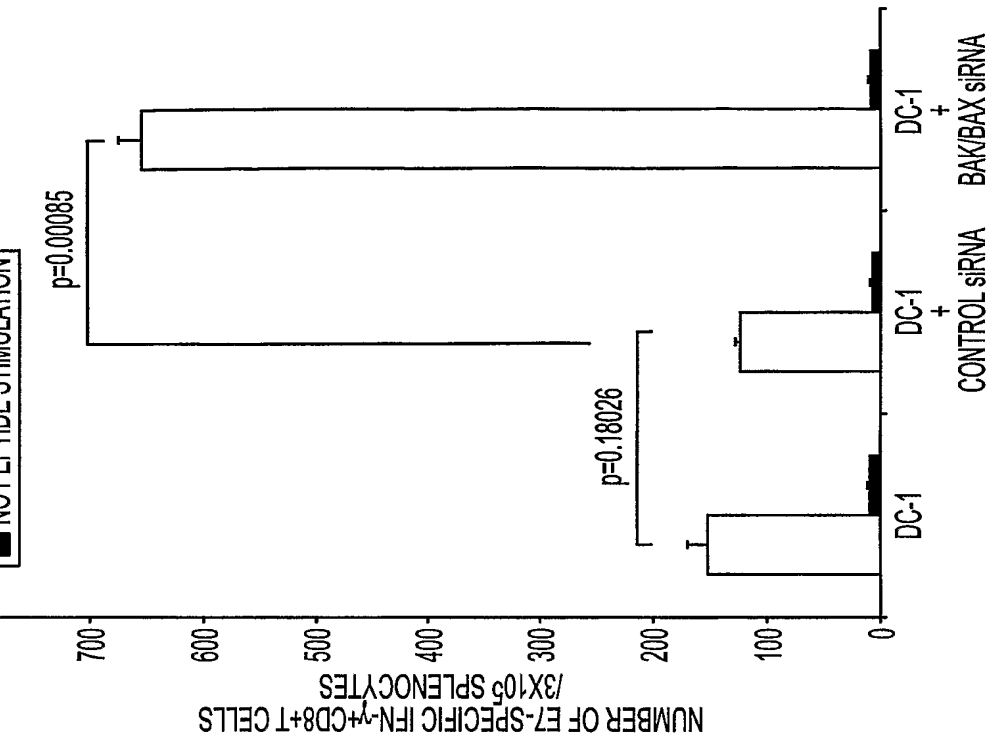
E7 aa49-57
NO PEPTIDE STIMULATION
p=0.00085
p=0.18026
NUMBER OF E7-SPECIFIC IFN-γ+CD8+T CELLS
/3X10^5 SPLENOCYTES
800
700
600
500
400
300
200
100
0
DC-1
DC-1 + CONTROL siRNA
DC-1 + BAK/BAX siRNA

FIG. 23

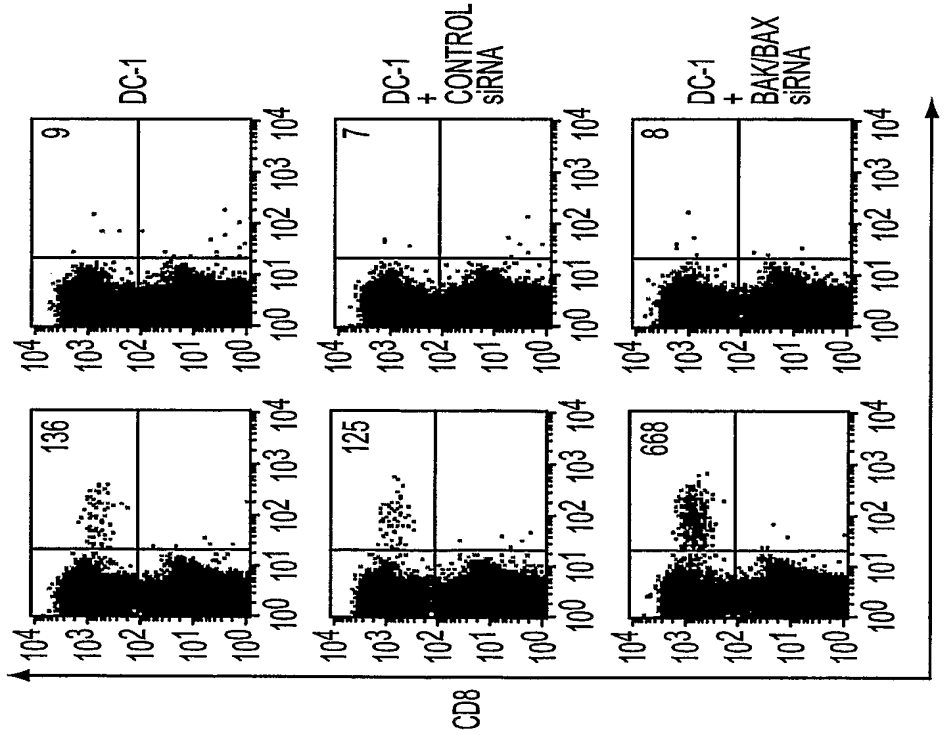
HPV-16 E7 aa49-57
NO PEPTIDE STIMULATION
DC-1
DC-1 + CONTROL siRNA
DC-1 + BAK/BAX siRNA
136
9
125
7
668
8
CD8
IFN-γ

FIG. 22

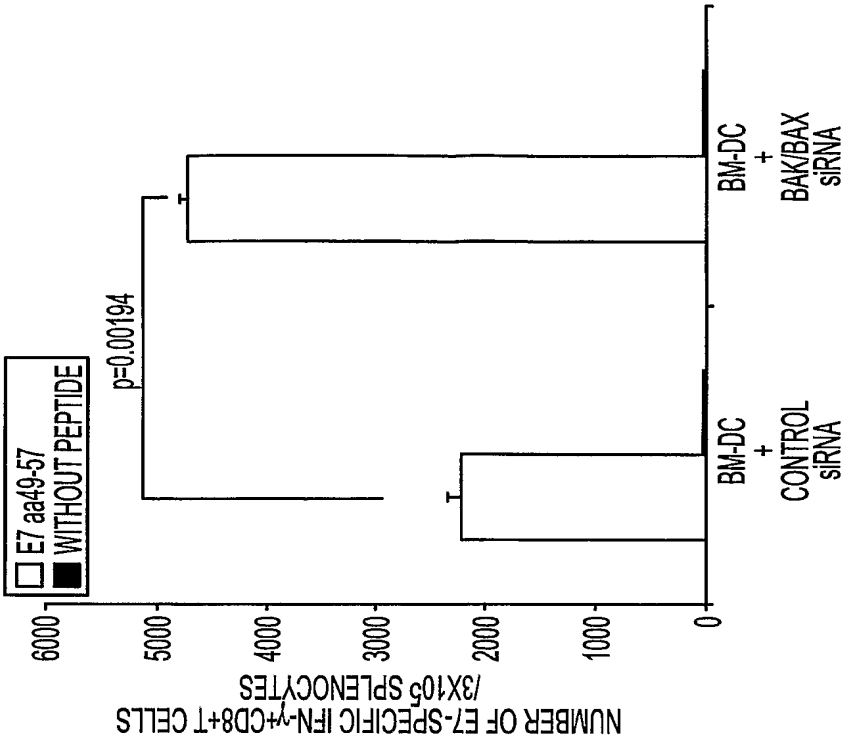
NUMBER OF E7-SPECIFIC IFN-γ+CD8+T CELLS
/3X10⁵ SPLENOCYTES
6000
5000
4000
3000
2000
1000
0
E7 aa49-57
WITHOUT PEPTIDE
p=0.00194
BM-DC + CONTROL siRNA
BM-DC + BAK/BAX siRNA

FIG. 25

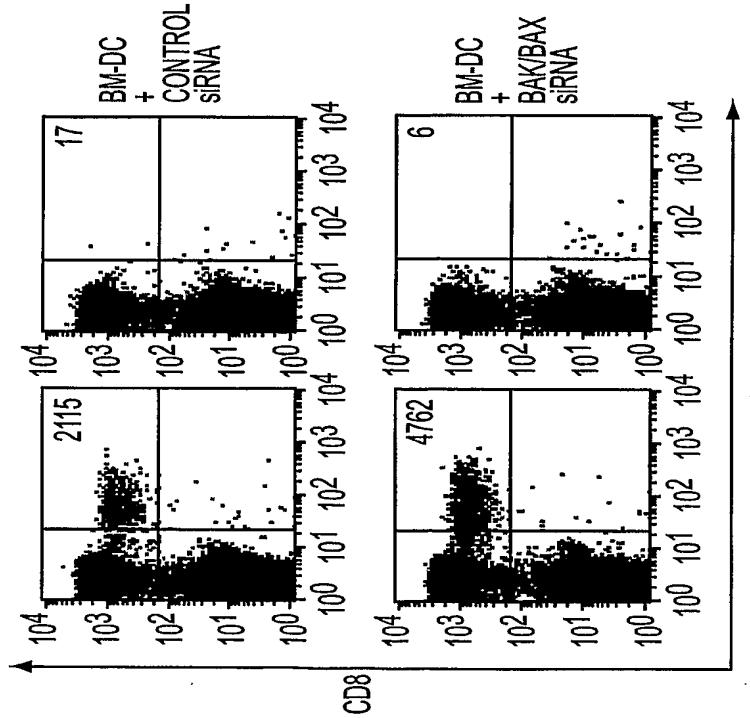
HPV-16 E7 aa49-57
NO PEPTIDE
BM-DC + CONTROL siRNA
BM-DC + BAK/BAX siRNA
2115
17
4762
6
CD8
IFN-γ

FIG. 24

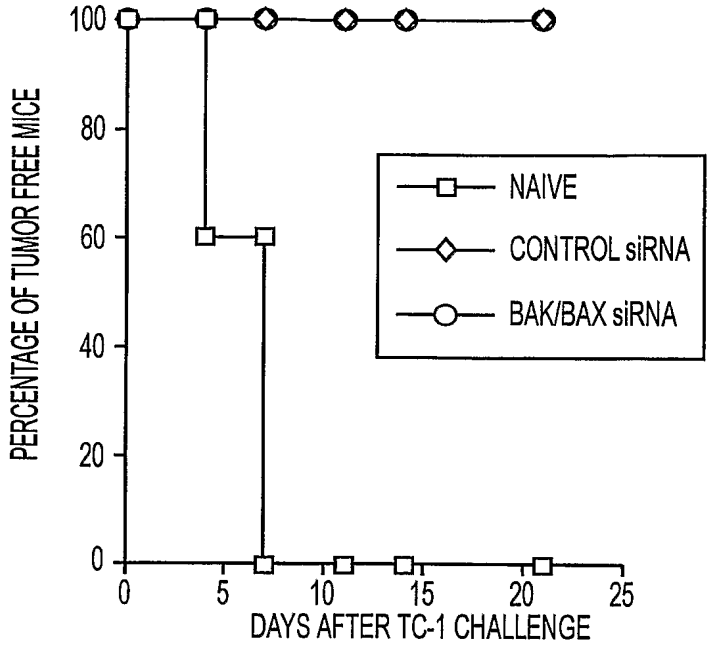
PERCENTAGE OF TUMOR FREE MICE
100
80
60
40
20
0
0
5
10
15
20
25
DAYS AFTER TC-1 CHALLENGE
NAIVE
CONTROL siRNA
BAK/BAX siRNA

FIG. 26

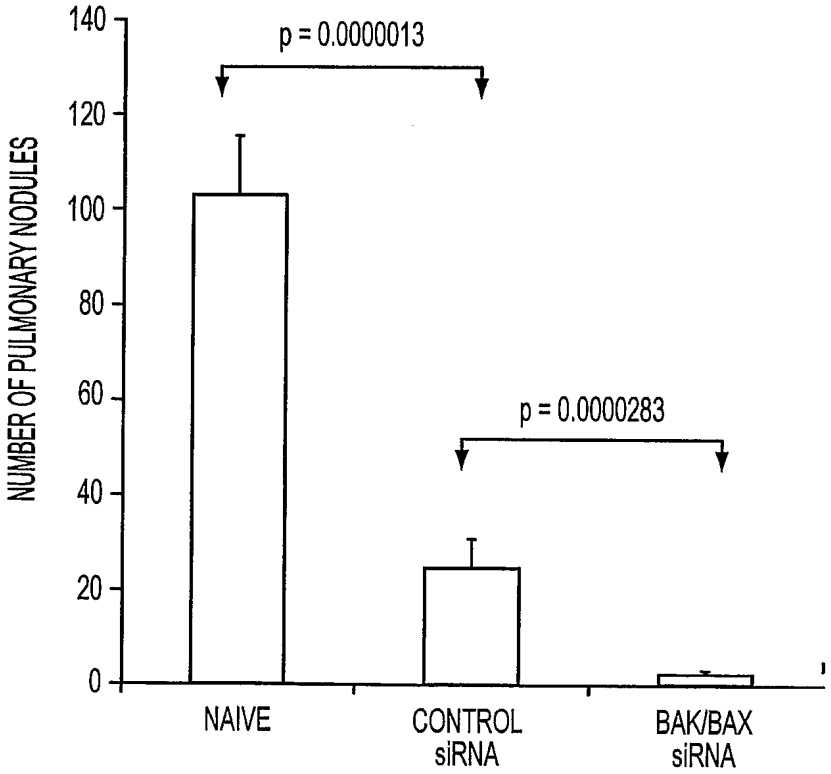
NUMBER OF PULMONARY NODULES
140
120
100
80
60
40
20
0
p = 0.0000013
p = 0.0000283
NAIVE
CONTROL siRNA
BAK/BAX siRNA

FIG. 27

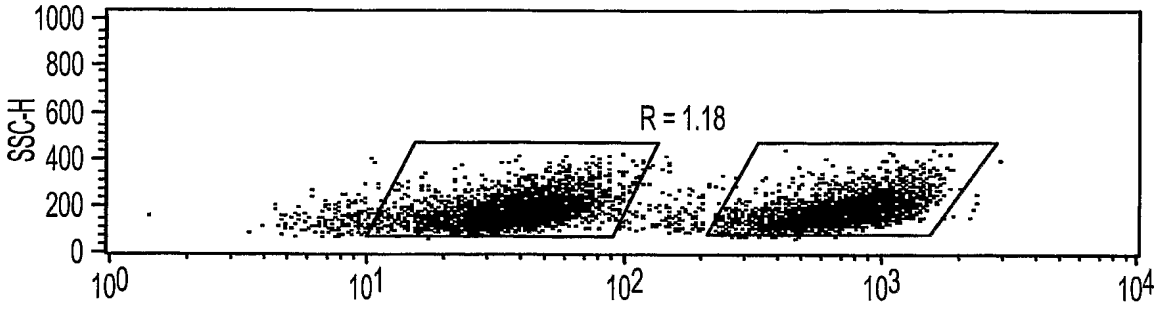

FIG. 28A

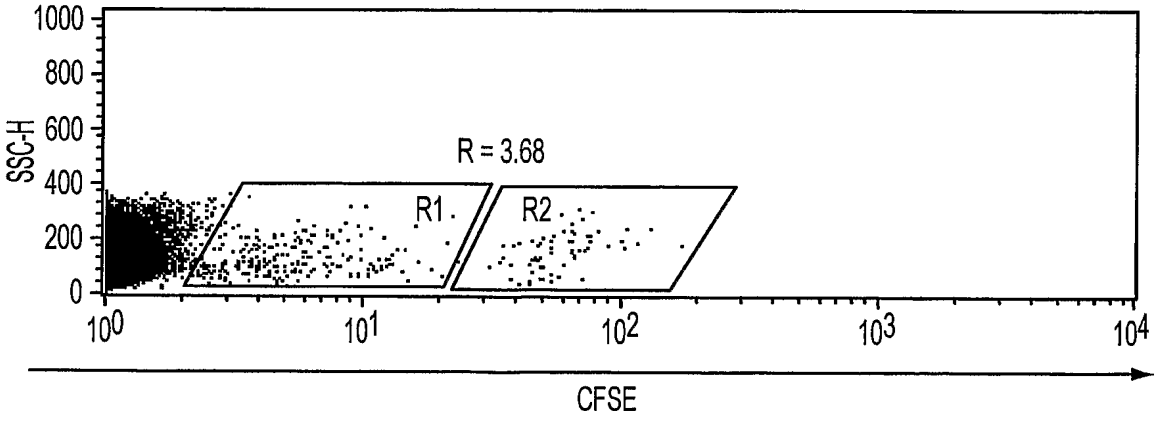

FIG. 28B

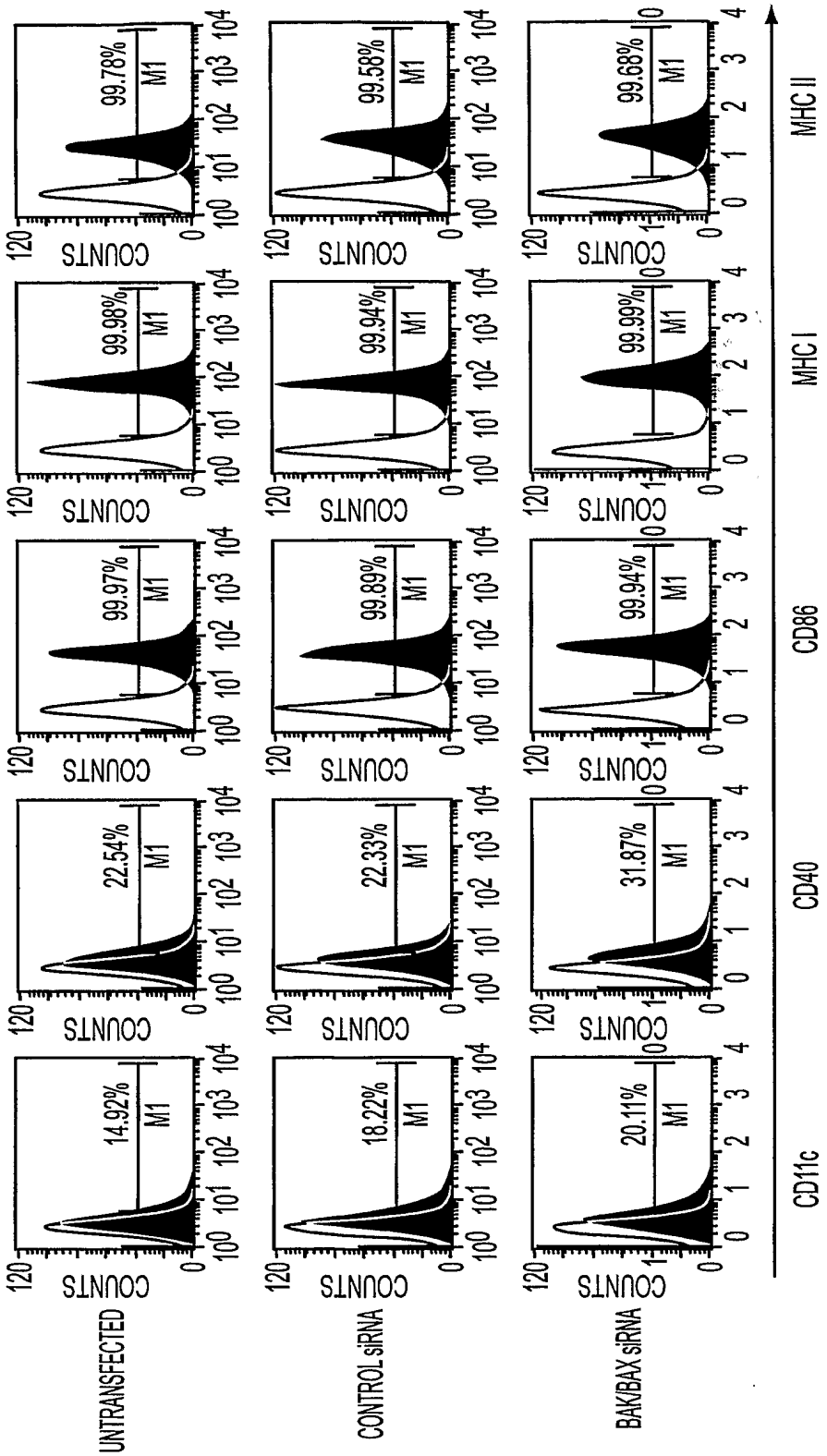
UNTRANSFECTED
CONTROL siRNA
BAK/BAX siRNA
CD11c
CD40
CD86
MHC I
MHC II
COUNTS
M1
14.92%
22.54%
99.97%
99.78%
18.22%
22.33%
99.89%
99.58%
20.11%
31.87%
99.94%
99.68%
99.98%
99.94%
99.99%

FIG. 29